CHAPTER 6
Electrophoresis and Immunoblotting

INTRODUCTION

The development of powerful new technologies is a major driving force of scientific progress. A good example of this is the role that electrophoretic techniques have played in the evolution of modern cell biology. Electrophoresis and related applications have contributed greatly to the understanding of the molecular bases of cell structure and function. The combination of high resolution, ease of use, speed, low cost, and versatility of electrophoretic techniques is unmatched by any other method used to separate proteins. It is for this reason that electrophoresis is an indispensable tool in any cell biology laboratory and that papers describing basic techniques of protein electrophoresis (e.g., Laemmli, 1970, and O'Farrell, 1975, to name just a couple) are among the most cited articles in this field. Laemmli's technique of discontinuous gel electrophoresis in the presence of SDS, for example, continues to be widely used and referenced almost 30 years after publication. Thus, no book of techniques in cell biology would be complete without a detailed description of electrophoretic techniques.

Chapter 6 begins with *UNIT 6.1*, which is a collection of state-of-the-art protocols for analyzing proteins by one-dimensional electrophoresis under denaturing conditions on polyacrylamide gels. Sodium dodecyl sulfate (SDS), in combination with a reducing agent and heat, is most often used as a denaturant. This type of electrophoresis is thus referred to as SDS-polyacylamide gel electrophoresis (SDS-PAGE). Denaturation of the proteins prior to electrophoresis allows for enhanced resolution and discrimination of proteins on the basis of molecular size rather than charge or shape. Utilization of a discontinuous system (i.e., the apposition of "stacking" and "separating" gels) results in concentration of dilute samples and enhanced band sharpness. *UNIT 6.1* presents an overview of electricity and electrophoresis, followed by detailed protocols for SDS-PAGE using either Laemmli's buffers and gel system or modifications of this system (i.e., use of Tris-tricine buffers, higher concentrations of buffers, gradient gels, single-concentration gels, and minigels). The unit also explains how to calculate the apparent molecular weights of proteins from SDS-PAGE data.

The next unit in the chapter, *UNIT 6.2*, describes protocols for immunoblotting (also referred to as western blotting). In this technique, proteins separated by any of the electrophoretic techniques described in *UNIT 6.1* are electrophoretically transferred ("electroblotted") onto a membrane. The membrane, which thus becomes a replica of the polyacrylamide gel, is subsequently probed with antibodies to specific proteins. The primary antibodies can be revealed by an additional incubation with ^{125}I-labeled secondary antibodies or protein A, followed by autoradiography (*UNIT 6.3*). In recent years, however, the use of radioiodinated antibodies has been progressively replaced by nonradioactive detection with antibodies coupled to enzymes such as alkaline phosphatase or horseradish peroxidase (see *UNIT 16.5*). The enzymes act on substrates which are converted to colored, luminescent, or fluorescent products. Nonradioactive methods are just as sensitive as radioactive methods, with the added advantage that they do not require the special precautions associated with the use of radioactivity. Nonradioactive detection is nowadays the method of choice for visualizing immunoblotted proteins. A disadvantage of nonradioactive methods is that they have a narrower linear range of detection, which can be a problem in experiments that require accurate quantitation of protein levels.

Contributed by Juan S. Bonifacino
Current Protocols in Cell Biology (2002) 6.0.1-6.0.3
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Radiolabeled proteins separated by electrophoresis or proteins detected by immunoblotting with radioiodinated antibodies or protein A can be visualized by autoradiography, as described in *UNIT 6.3*. In this technique, ionizing radiation emanating from the radionuclides impresses a photographic film. The technique can be made more sensitive by the use of intensifying screens or scintillating compounds, which emit light upon radiation absorption, which then impresses the film. The unit contains several protocols for autoradiographic detection of various radionuclides, including methods for enhancing the signal with intensifying screens or by fluorography. Also included in *UNIT 6.3* are discussions of the quantification of film images by densitometry and the direct detection and quantification of radioactive samples in gels by phosphor imaging.

The resolution of electrophoretic techniques can be enormously enhanced by combining two different electrophoretic procedures performed successively in perpendicular directions (i.e., two-dimensional gel electrophoresis). The most common type of two-dimensional gel electrophoresis is based on separation of proteins by isoelectric focusing on a tube gel (first dimension) followed by SDS-PAGE on a slab gel (second dimension). The two processes separate proteins on the basis of charge and size, respectively, allowing resolution of up to several thousand proteins on a single two-dimensional gel. *UNIT 6.4* describes several methods for separating proteins by two-dimensional isoelectric focusing/SDS-PAGE. In addition, this unit presents a protocol for two-dimensional nonreducing/reducing electrophoresis in which proteins are separated by SDS-PAGE under nonreducing conditions in the first dimension and under reducing conditions in the second dimension. This type of two-dimensional gel electrophoresis allows analysis of intersubunit disulfide bonds in multiprotein complexes and, in some cases, of intrasubunit disulfide bonds. Both types of two-dimensional gel electrophoresis can be used for either analytical or preparative purposes.

Another useful method for electrophoretic separation of cellular proteins is one-dimensional electrophoresis under nondenaturing conditions. Two protocols describing variations of this method are included in *UNIT 6.5*. What distinguishes this method from those described in *UNIT 6.1* and *UNIT 6.4* is that protein samples are not exposed to denaturing agents (i.e., SDS or urea) either prior to or during electrophoresis. Thus, proteins migrate according to their native properties, such as size, shape, and charge. This allows analysis of the oligomeric state of proteins, conformational changes, charge heterogeneity, and post-translational modifications that affect conformation or charge while having minimal effects on the molecular weights of the proteins. In many cases, this method preserves the intrinsic function of the proteins, which allows their detection with specific activity or binding assays. The first protocol describes continuous electrophoresis on nondenaturing polyacrylamide gels. This system involves electrophoresis on a single separating gel and uses the same buffer in the chambers and the gel. The second protocol, discontinuous electrophoresis on nondenaturing polyacrylamide gels, is a variation of SDS-PAGE in which SDS and reducing agents are omitted from all the solutions. Determination of the migration of proteins on parallel gels made up of different concentrations of acrylamide and bisacrylamide allows calculation of their molecular weights using Ferguson plots.

Proteins separated by electrophoresis can be visualized by direct staining of the gels. Four procedures for staining proteins in gels based on different principles are presented in *UNIT 6.6*. These procedures involve staining with Coomassie blue, silver, SYPRO ruby or zinc ions. The unit describes the basic protocols and provides guidelines for the selection of a specific protocol.

The separation of proteins by polyacrylamide gel electrophoresis is limited to proteins with molecular weights less than ~300,000. The electrophoretic separation of larger proteins or multiprotein complexes requires the use of other matrix materials. A suitable material is agarose, which is most commonly used for the separation of DNA. *UNIT 6.7* presents protocols for the electrophoretic separation of proteins on agarose gels. In

addition to permitting the separation of very large proteins, these protocols allow the analysis of multimerization or aggregation. Although these processes can also be analyzed by sedimentation on sucrose gradients (*UNIT 6.3*) or gel filtration (*UNIT 6.4*), agarose gel electrophoresis is more convenient for analysis of large numbers of samples.

LITERATURE CITED

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One-Dimensional SDS Gel Electrophoresis of Proteins

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ABSTRACT

Electrophoresis is used to separate complex mixtures of proteins (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates), to investigate subunit compositions, and to verify homogeneity of protein samples. It can also serve to purify proteins for use in further applications. In polyacrylamide gel electrophoresis, proteins migrate in response to an electrical field through pores in a polyacrylamide gel matrix; pore size decreases with increasing acrylamide concentration. The combination of pore size and protein charge, size, and shape determines the migration rate of the protein. In this unit, the standard Laemmli method is described for discontinuous gel electrophoresis under denaturing conditions, that is, in the presence of sodium dodecyl sulfate (SDS). Both full-size and minigel formats are detailed. Several modifications are provided for specific applications. For separation of peptides and small proteins, the standard buffers are replaced with either a Tris-tricine buffer system or a modified Tris buffer in the absence of urea. Continuous SDS-PAGE is a simplified method in which the same buffer is used for both the gel and the electrode solutions and the stacking gel is omitted. Other protocols cover the preparation and use of ultrathin gels and gradient gels, and the simultaneous preparation of multiple gels. *Curr. Protoc. Cell Biol.* 37:6.1.1-6.1.38. © 2007 by John Wiley & Sons, Inc.

Keywords: protein • electrophoresis • separation • polyacrylamide • SDS-PAGE

INTRODUCTION

Electrophoresis is used to separate complex mixtures of proteins (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates), to investigate subunit compositions, and to verify homogeneity of protein samples. It can also serve to purify proteins for use in further applications. In polyacrylamide gel electrophoresis (PAGE), proteins migrate in response to an electrical field through pores in the gel matrix; pore size decreases with higher acrylamide concentrations. The combination of gel pore size and protein charge, size, and shape determines the migration rate of the protein.

The standard Laemmli method (see Basic Protocol 1) is used for discontinuous gel electrophoresis under denaturing conditions, that is, in the presence of sodium dodecyl sulfate (SDS). The standard method for full-size gels (e.g., 14 × 14 cm) can be adapted for the minigel format (e.g., 7.3 × 8.3 cm; see Basic Protocol 2). Minigels provide rapid separation but give lower resolution.

Several alternate protocols are provided for specific applications. The first two alternate protocols cover electrophoresis of peptides and small proteins, separations that require modification of standard buffers: either a Tris-tricine buffer system (see Alternate Protocol 1) or a modified Tris buffer in the absence of urea (see Alternate Protocol 2). Continuous SDS-PAGE is a simplified method in which the same buffer is used for both gel and electrode solutions and the stacking gel is omitted (see Alternate Protocol 3). Other protocols cover the preparation and electrophoresis of various types of gels:

Current Protocols in Cell Biology 6.1.1-6.1.38, December 2007
Published online December 2007 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0601s37
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ultrathin gels (see Alternate Protocol 4), multiple single-concentration gels (see Support Protocol 1), gradient gels (see Alternate Protocol 5), multiple gradient gels (see Support Protocol 2), and multiple gradient minigels (see Support Protocol 3). Proteins separated on gels can be subsequently analyzed by immunoblotting (*UNIT 6.2*), autoradiography or phosphor imaging (*UNIT 6.3*), or staining with protein dyes (*UNIT 6.6*). Protein size is determined by comparing the mobility of the protein band to the mobility of the dye front (see Support Protocol 4).

CAUTION: Before any protocols are used, it is extremely important to read the following section about electricity and electrophoresis.

ELECTRICITY AND ELECTROPHORESIS

Many researchers are poorly informed concerning the electrical parameters of running a gel. It is important to note that the voltages and currents used during electrophoresis are dangerous and potentially lethal. Thus, safety should be an overriding concern. A working knowledge of electricity is an asset in determining what conditions to use and in troubleshooting the electrophoretic separation, if necessary. For example, an unusually high or low voltage for a given current (milliampere) might indicate an improperly made buffer or an electrical leak in the chamber.

Safety Considerations

1. Never remove or insert high-voltage leads unless the power supply voltage is turned down to zero and the power supply is turned off. Always grasp high-voltage leads one at a time with one hand only. Never insert or remove high-voltage leads with both hands. This can shunt potentially lethal electricity through the chest and heart should electrical contact be made between a hand and a bare wire. On older or homemade instruments, the banana plugs may not be shielded and can still be connected to the power supply at the same time they make contact with a hand. With commercial modern power supplies, this is less of an issue. However, with age and use, wires may become exposed through cracks in the insulation or poor connections. Carefully inspect all cables and connections and replace frayed or exposed wires immediately.

2. Always start with the power supply turned off. Have the power supply controls turned all the way down to zero. Then hook up the gel apparatus: generally, connect the red high-voltage lead to the red outlet and the black high-voltage lead to the black outlet. Turn the power supply on with the controls set at zero and the high-voltage leads connected. Then turn up the voltage, current, or power to the desired level. Reverse the process when the power supply is turned off: i.e., to disconnect the gel, turn the power supply down to zero, wait for the meters to read zero, turn off the power supply, and then disconnect the gel apparatus one lead at a time.

   CAUTION: *If the gel is first disconnected and then the power supply turned off, a considerable amount of electrical charge is stored internally. The charge will stay in the power supply over a long time. This will discharge through the outlets even though the power supply is turned off and can deliver an electrical shock.*

Ohm's Law and Electrophoresis

Understanding how a gel apparatus is connected to the power supply requires a basic understanding of Ohm's law: voltage = current × resistance, or $V = IR$. A gel can be viewed as a resistor and the power supply as the voltage and current source. Most power supplies deliver constant current or constant voltage. Some will also deliver constant power: power = voltage × current, or $VI = I^2R$. The discussion below focuses on constant current because this is the most common mode in vertical SDS-PAGE.

Most modern commercial equipment is color-coded so that the red or positive terminal of the power supply can simply be connected to the red lead of the gel apparatus, which goes to the lower buffer chamber. The black lead is connected to the black or negative terminal and goes to the upper buffer chamber. This configuration is designed to work with vertical slab gel electrophoreses in which negatively charged proteins or nucleic acids move to the positive electrode in the lower buffer chamber (an anionic system).

When a single gel is attached to a power supply, the negative charges flow from the negative cathode (black) terminal into the upper buffer chamber, through the gel, and into the lower buffer chamber. The lower buffer chamber is connected to the positive anode (red) terminal to complete the circuit. Thus, negatively charged molecules, such as SDS-coated proteins and nucleic acids, move from the negative cathode attached to the upper buffer chamber toward the positive anode attached to the lower chamber. SDS-PAGE is an anionic system because of the negatively charged SDS.

Occasionally, proteins are separated in cationic systems. In these gels, the proteins are positively charged because of the very low pH of the gel buffers (e.g., acetic acid/urea gels for histone separations) or the presence of a cationic detergent (e.g., cetyltrimethyl-ammonium bromide, CTAB). Proteins move toward the negative electrode (cathode) in cationic gel systems, and the polarity is reversed compared to SDS-PAGE: the red lead from the lower buffer chamber is attached to the black outlet of the power supply, and the black lead from the upper buffer chamber is attached to the red outlet of the power supply.

Most SDS-PAGE separations are performed under constant current (consult instructions from the manufacturer to set the power supply for constant current operation). The resistance of the gel will increase during SDS-PAGE in the standard Laemmli system. If the current is constant, then the voltage will increase during the run as the resistance goes up.

Power supplies usually have more than one pair of outlets. The pairs are connected in parallel with one another internally. If more than one gel is connected directly to the outlets of a power supply, then these gels are connected in parallel (Fig. 6.1.1). In a parallel circuit, the voltage is the same across each gel. In other words, if the power supply reads 100 V, then each gel has 100 V across its electrodes. The total current, however, is the sum of the individual currents going through each gel. Therefore, under constant current it is necessary to increase the current for each additional gel that is connected to the power supply. Two identical gels require double the current to achieve the same starting voltages and electrophoresis separation times.

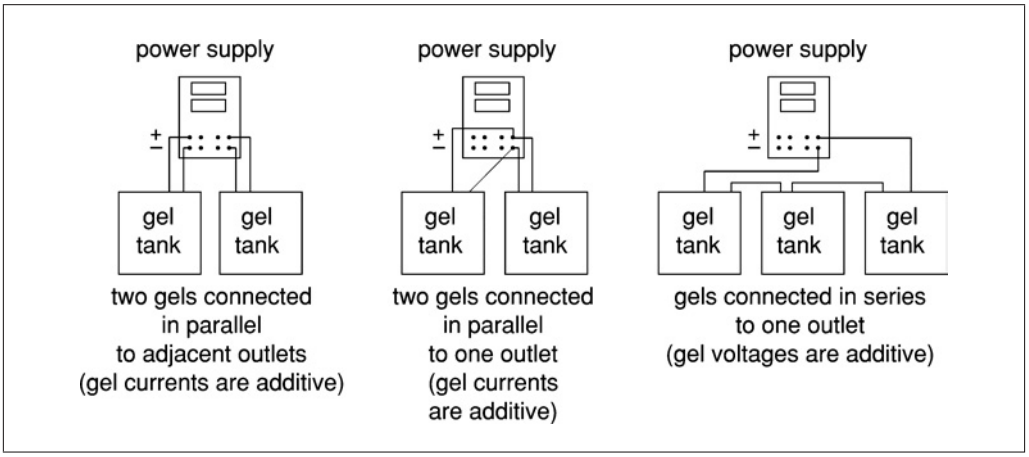

Figure 6.1.1 Series and parallel connections of gel tanks to power supply.

Multiple gel apparatuses can also be connected to one pair of outlets on a power supply. This is useful with older power supplies that have a limited number of outlets. When connecting several gel units to one outlet, make certain the connections between the units are shielded and protected from moisture. The gels can be connected in parallel or in series (Fig. 6.1.1). When gels are connected through the same outlet in parallel, the principle of additive currents is the same as for gels connected through different outlets in parallel. In the case of two or more gels running off the same outlet in series, the current is the same for every gel. If 10 mA is displayed by the power supply meter, for example, each gel in series will experience 10 mA. The voltage, however, is additive for each gel. If one gel at a constant 10 mA produces 100 V, then two identical gels in series will produce 200 V (100 V each) and so on. Thus, the voltage can limit the number of units connected in series on low-voltage power supplies.

Gel thickness affects the above relationships. A 1.5-mm gel can be thought of as two 0.75-mm-thick gels run in parallel. Because currents are additive in parallel circuits, a 0.75-mm gel will require half the current of the 1.5-mm gel to achieve the same starting voltage and separation time. If gel thickness is doubled, then the current must also be doubled. There are limits to the amount of current that can be applied. Thicker gels require more current, generating more heat that must be dissipated. Unless temperature control is available in the gel unit, a thick gel should be run more slowly than a thin gel.

NOTE: Milli-Q-purified water or equivalent should be used throughout the protocols.

BASIC PROTOCOL 1

DENATURING (SDS) DISCONTINUOUS GEL ELECTROPHORESIS: LAEMMLI GEL METHOD

One-dimensional gel electrophoresis under denaturing conditions (i.e., in the presence of 0.1% SDS) separates proteins based on molecular size as they move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel (sometimes called resolving or running gel) topped by a stacking gel and secured in an electrophoresis apparatus. After sample proteins are solubilized by boiling in the presence of SDS, an aliquot of the protein solution is applied to a gel lane, and the individual proteins are separated electrophoretically. The stacking gel, through a combination of low porosity and a lower buffer concentration and pH, concentrates proteins into a thin stack before they enter the resolving gel. 2-Mercaptoethanol (2-ME) or dithiothreitol (DTT) is added during solubilization to reduce disulfide bonds.

This protocol is designed for a vertical slab gel with a maximum size of 0.75 mm × 14 cm × 14 cm. For thicker gels or minigels (see Basic Protocol 2 and Support Protocol 3), the volumes of stacking and separating gels and the operating current must be adjusted. Additional protocols describe the preparation of ultrathin gels (see Alternate Protocol 4) and gradient gels (see Alternate Protocol 5), as well as the use of gel casters to make multiple gels, both single-concentration gels (see Support Protocol 1) and gradient gels (see Support Protocol 2).

Materials

Separating and stacking gel solutions (Table 6.1.1)
H_2O-saturated isobutyl alcohol
1× Tris·Cl/SDS, pH 8.8 (dilute 4× Tris·Cl/SDS, pH 8.8; Table 6.1.1)
Protein sample, on ice
2× and 1× SDS sample buffer (see recipe)
Protein molecular weight standards (Tables 6.1.2 and 6.1.3)
6× SDS sample buffer (see recipe; optional)
1× SDS electrophoresis buffer (see recipe)

Electrophoresis apparatus: e.g., Protean II 16-cm cell (Bio-Rad) or SE 600/400 16-cm unit (Hoefer) with clamps, glass plates, casting stand, and buffer chambers
0.75-mm spacers
0.45-μm filters (used in stock solution preparation)
25-ml Erlenmeyer side-arm flasks
Vacuum pump with cold trap
0.75-mm Teflon comb with 1, 3, 5, 10, 15, or 20 teeth
Screw-top microcentrifuge tubes (recommended)
25- or 100-μl syringe with flat-tipped needle
Constant-current power supply (see Electricity and Electrophoresis above)

Pour the separating gel

1. Assemble the glass-plate sandwich of the electrophoresis apparatus according to manufacturer's instructions using two clean glass plates and two 0.75-mm spacers.

   If needed, clean the glass plates in liquid Alconox or RBS-35 (Pierce). These aqueous-based solutions are compatible with silver and Coomassie blue staining procedures.

2. Lock the sandwich to the casting stand.

3. Prepare the separating gel solution as directed in Table 6.1.1, degassing using a rubber-stoppered 25-ml Erlenmeyer side-arm flask connected with vacuum tubing to a vacuum pump with a cold trap. After adding the specified amount of 10% ammonium persulfate and TEMED to the degassed solution, stir gently to mix.

   The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. Generally, use 5% gels for SDS-denatured proteins of 60 to 200 kDa, 10% gels for SDS-denatured proteins of 16 to 70 kDa, and 15% gels for SDS-denatured proteins of 12 to 45 kDa (Table 6.1.1).

   The stacking gel is the same regardless of the separating gel used.

4. Using a Pasteur pipet, apply the separating gel solution to the sandwich along an edge of one of the spacers until the height of the solution between the glass plates is ~11 cm.

   Use the solution immediately; otherwise it will polymerize in the flask.

   Sample volumes <10 μl do not require a stacking gel. In this case, cast the resolving gel as usual, but extend the resolving gel into the comb (step 10) to form the wells. The proteins are then separated under the same conditions as used when a stacking gel is present. Although this protocol works well with single-concentration gels, a gradient gel is recommended for maximum resolution (see Alternate Protocol 5).

5. Using another Pasteur pipet, slowly cover the top of the gel with a layer (~1 cm thick) of H_2O-saturated isobutyl alcohol, by gently layering the isobutyl alcohol against the edge of one and then the other of the spacers.

   Be careful not to disturb the gel surface. The overlay provides a barrier to oxygen, which inhibits polymerization, and allows a flat interface to form during gel formation.

   The H_2O-saturated isobutyl alcohol is prepared by shaking isobutyl alcohol and H_2O in a separatory funnel. The aqueous (lower) phase is removed. This procedure is repeated several times. The final upper phase is H_2O-saturated isobutyl alcohol.

6. Allow the gel to polymerize 30 to 60 min at room temperature.

   A sharp optical discontinuity at the overlay/gel interface will be visible on polymerization. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both. Ammonium persulfate solution should be made fresh before use. Ammonium persulfate should "crackle" when added to the water. If not, fresh ammonium persulfate should be purchased. Purchase TEMED in small bottles so, if necessary, a new previously unopened source can be tried.

Table 6.1.1 Recipes for Polyacrylamide Separating and Stacking Gels[a]

SEPARATING GEL

Stock solution[b]	Final acrylamide concentration in separating gel (%)[c]									
	5	6	7	7.5	8	9	10	12	13	15
30% (w/v) acrylamide/ 0.8% (w/v) bisacrylamide	2.50	3.00	3.50	3.75	4.00	4.50	5.00	6.00	6.50	7.50
4× Tris·Cl/SDS, pH 8.8	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.50	7.25	6.75	6.25	5.25	4.75	3.75
10% (w/v) ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED[e]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Preparation of separating gel

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution, 4× Tris·Cl/SDS, pH 8.8 (see reagents, below), and H_2O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

STACKING GEL (*3.9% w/v acrylamide*)

In a 25-ml side-arm flask, mix 0.65 ml of 30% acrylamide/0.8% bisacrylamide, 1.25 ml of 4× Tris·Cl/SDS, pH 6.8 (see reagents, below), and 3.05 ml H_2O. Degas under vacuum 10 to 15 min. Add 25 µl of 10% ammonium persulfate and 5 µl TEMED. Swirl gently to mix. Use immediately.

REAGENTS USED IN GELS

30% (w/v) acrylamide/0.8% (w/v) bisacrylamide

Mix 30.0 g acrylamide and 0.8 g *N,N′*-methylenebisacrylamide with H_2O in a total volume of 100 ml. Filter the solution through a 0.45-µm filter and store at 4°C in the dark. The 2× crystallized grades of acrylamide and bisacrylamide are recommended. Discard after 30 days, as acrylamide gradually hydrolyzes to acrylic acid and ammonia.

CAUTION: Acrylamide monomer is neurotoxic. A mask should be worn when weighing acrylamide powder. Gloves should be worn while handling the solution, and the solution should not be pipetted by mouth.

4× Tris·Cl/SDS, pH 6.8 *(0.5 M Tris·Cl containing 0.4% w/v SDS)*

Dissolve 6.05 g Tris base in 40 ml H_2O. Adjust to pH 6.8 with 1 N HCl. Add H_2O to 100 ml total volume. Filter the solution through a 0.45-µm filter, add 0.4 g SDS, and store at 4°C up to 1 month.

4× Tris·Cl/SDS, pH 8.8 *(1.5 M Tris·Cl containing 0.4% w/v SDS)*

Dissolve 91 g Tris base in 300 ml H_2O. Adjust to pH 8.8 with 1 N HCl. Add H_2O to 500 ml total volume. Filter the solution through a 0.45-µm filter, add 2 g SDS, and store at 4°C up to 1 month.

[a]The recipes produce 15 ml of separating gel and 5 ml of stacking gel, which are adequate for a gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the SDS (denaturing) discontinuous buffer system of Laemmli (1970).

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Volumes are in milliliters. The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. See annotation to step 3, Basic Protocol 1.

[d]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

[e]TEMED, *N,N,N,N*-tetramethylethylenediamine.

Table 6.1.2 Molecular Weights of Protein Standards for Polyacrylamide Gel Electrophoresis[a]

Protein	Molecular weight (Da)
Cytochrome *c*	11,700
α-Lactalbumin	14,200
Lysozyme (hen egg white)	14,300
Myoglobin (sperm whale)	16,800
β-Lactoglobulin	18,400
Trypsin inhibitor (soybean)	20,100
Trypsinogen, PMSF treated	24,000
Carbonic anhydrase (bovine erythrocytes)	29,000
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)	36,000
Lactate dehydrogenase (porcine heart)	36,000
Aldolase	40,000
Ovalbumin	45,000
Catalase	57,000
Bovine serum albumin	66,000
Phosphorylase *b* (rabbit muscle)	97,400
β-Galactosidase	116,000
RNA polymerase, *E. coli*	160,000
Myosin, heavy chain (rabbit muscle)	205,000

[a]Protein standards are commercially available as prepared mixtures (see Table 6.1.3).

Pour the stacking gel

7. Pour off the layer of H_2O-saturated isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8.

   Residual isobutyl alcohol can reduce resolution of the protein bands; therefore, it must be completely removed. The isobutyl alcohol overlay should not be left on the gel longer than 2 hr.

8. Prepare the stacking gel solution as directed in Table 6.1.1.

   Use the solution immediately to keep it from polymerizing in the flask.

9. Using a Pasteur pipet, allow the stacking gel solution to trickle slowly into the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich is ~1 cm from the top of the plates.

   Be careful not to introduce air bubbles into the stacking gel.

10. Insert a 0.75-mm Teflon comb into the layer of stacking gel solution. If necessary, add additional stacking gel to fill the spaces in the comb completely.

    Again, be careful not to trap air bubbles in the tooth edges of the comb; they will cause small circular depressions in the well after polymerization that will lead to distortion in the protein bands during separation.

11. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

    A sharp optical discontinuity will be visible around the wells on polymerization. Again, failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

Table 6.1.3 Protein Standard Mixtures Available from Selected Suppliers

Applications[a]	1-D	2-D[b]	Im	Pre[c]	Fluor	Gly	Phos	Bio	Tag	IEF	Nat
Bio-Rad	X	X		X				X	X	X	
CalBiochem	X						X				
Cell Signaling Technology	X			X				X			
Favorgen	X			X							
GE Healthcare	X		X	X				X	X	X	
Invitrogen	X		X	X	X	X	X		X	X	
NEB	X			X							
Norgen Biotek	X		X								
Novagen	X		X	X					X		
PerkinElmer	X			X							
Pierce	X	X		X	X						
Promega	X										
Qiagen	X		X						X		
R & D Systems	X			X				X			
Roche Applied Science	X								X		
Sigma-Aldrich	X	X	X	X	X	X	X	X		X	X
Upstate	X		X				X				
USB	X		X							X	

[a]Abbreviations: 1-D, one-dimensional gels; 2-D, two-dimensional gels; Im, immunoblotting; Pre, prestained; Fluor, fluorescent; Gly, glycoprotein; Phos, phosphoprotein; Bio, biotinylated; Tag, tagged; IEF, isoelectic focusing; Nat, native.

[b]2-D standards are useful as independently characterized internal controls or reference standards for 2-D SDS-PAGE. Many investigators simply use an internally characterized test sample as a reference set.

[c]Prestained standards, while not as sharply delineated as unstained standards, can be used to monitor progress of the separation since the bands are visible through the gel cassette during electrophoresis. They are also useful for marking the position of a band after electroblotting to a nitrocellulose or PVDF membrane prior to immunoassay or analysis by mass spectrometry.

Prepare the sample and load the gel

12. Dilute a portion of the protein sample to be analyzed 1:1 (v/v) with 2× SDS sample buffer and heat 3 to 5 min at 100°C in a sealed screw-cap microcentrifuge tube. If the sample is a precipitated protein pellet, dissolve the protein in 50 to 100 µl of 1× SDS sample buffer and boil 3 to 5 min at 100°C. Dissolve protein molecular weight standards in 1× SDS sample buffer according to supplier's instructions; use these standards as a control (Tables 6.1.2 and 6.1.3).

    For dilute protein solutions, consider using 5:1 protein solution/6× SDS sample buffer to increase the amount of protein loaded. Proteins can also be concentrated by precipitation in acetone, ethanol, or trichloroacetic acid (TCA), but losses will occur.

    For a 0.8-cm-wide well, 25 to 50 µg total protein in <20 µl is recommended for a complex mixture when staining with Coomassie blue, and 1 to 10 µg total protein is needed for samples containing one or a few proteins. If silver staining is used, 10- to 100-fold less protein can be applied (0.01 to 5 µg in <20 µl depending on sample complexity).

    To achieve the highest resolution possible, the following precautions are recommended. Prior to adding the sample buffer, keep samples at 0°C. Add the SDS sample buffer (room temperature) directly to the 0°C sample (still on ice) in a screw-top microcentrifuge tube.

Cap the tube to prevent evaporation, vortex, and transfer directly to a 100°C water bath for 3 to 5 min. Let immunoprecipitates dissolve for 1 hr at 56°C in 1× SDS sample buffer prior to boiling. DO NOT leave the sample in SDS sample buffer at room temperature without first heating to 100°C to inactivate proteases (see Critical Parameters and Troubleshooting). Endogenous proteases are very active in SDS sample buffer and will cause severe degradation of the sample proteins after even a few minutes at room temperature. To test for possible proteases, mix the sample with SDS sample buffer without heating and leave at room temperature for 1 to 3 hr. A loss of high-molecular-weight bands and a general smearing of the banding pattern indicate a protease problem. Once heated, the samples can sit at room temperature for the time it takes to load samples.

13. Carefully remove the Teflon comb without tearing the edges of the polyacrylamide wells. After the comb is removed, rinse wells with 1× SDS electrophoresis buffer.

    The rinse removes unpolymerized monomer; otherwise, the monomer will continue to polymerize after the comb is removed, creating uneven wells that will interfere with sample loading and subsequent separation.

14. Using a Pasteur pipet, fill the wells with 1× SDS electrophoresis buffer.

    If well walls are not upright, they can be manipulated with a flat-tipped needle attached to a syringe.

15. Attach gel sandwich to upper buffer chamber following manufacturer's instructions.

16. Fill lower buffer chamber with the recommended amount of 1× SDS electrophoresis buffer.

17. Place sandwich attached to upper buffer chamber into lower buffer chamber.

18. Partially fill the upper buffer chamber with 1× SDS electrophoresis buffer so that the sample wells of the stacking gel are filled with buffer.

    Monitor the upper buffer chamber for leaks and, if necessary, reassemble the unit. A slow leak in the upper buffer chamber may cause arcing around the upper electrode and damage the upper buffer chamber.

19. Using a 25- or 100-µl syringe with a flat-tipped needle, load the protein sample(s) into one or more wells by carefully applying the sample as a thin layer at the bottom of the wells. Load control wells with molecular weight standards. Add an equal volume of 1× SDS sample buffer to any empty wells to prevent spreading of adjoining lanes.

    Disposable loading tips can be used with automatic pipettors to simplify loading.

    Preparing the samples at approximately the same concentration and loading an equal volume to each well will ensure that all lanes are the same width and that the proteins run evenly. If unequal volumes of sample buffer are added to wells, the lane with the larger volume will spread during electrophoresis and constrict the adjacent lanes, causing distortions.

    The samples will layer on the bottom of the wells because the glycerol added to the sample buffer gives the solution a greater density than the electrophoresis buffer. To keep bands tight, hold the tip of the needle near the bottom of the well and load the samples slowly. The bromphenol blue in the sample buffer makes sample application easy to follow visually.

20. Fill the remainder of the upper buffer chamber with additional 1× SDS electrophoresis buffer so that the upper platinum electrode is completely covered. Do this slowly so that samples are not swept into adjacent wells.

Run the gel

21. Connect the power supply to the cell and run at 10 mA of constant current for a slab gel 0.75 mm thick, until the bromphenol blue tracking dye enters the separating gel. Then increase the current to 15 mA.

    For a standard 16-cm gel sandwich, 4 mA per 0.75-mm-thick gel will run ~15 hr (i.e., overnight); 15 mA per 0.75-mm gel will take 4 to 5 hr. To run two gels or a 1.5-mm-thick gel, simply double the current. When running a 1.5-mm gel at 30 mA, the temperature must be controlled (10° to 20°C) with a circulating constant-temperature water bath to prevent "smiling" (curvature in the migratory band). Temperatures <5°C should not be used because SDS in the running buffer will precipitate.

    If the level of buffer in the upper chamber decreases, a leak has occurred.

22. After the bromphenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

    Refer to Safety Considerations under Electricity and Electrophoresis.

Disassemble the gel

23. Discard electrophoresis buffer and remove the upper buffer chamber with the attached gel sandwich.

24. Orient the gel so that the order of the sample wells is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels.

25. Carefully slide one of the spacers halfway from the edge of the sandwich along its entire length. Use the exposed spacer as a lever to pry open the glass plate, exposing the gel.

26. Carefully remove the gel from the lower plate. Cut a small triangle off one corner of the gel so the lane orientation is not lost during staining and drying. Proceed with protein detection.

    Gradient gels are most easily picked up without tearing from the high-concentration end of the gel using gloved fingers. Single-concentration gels <10% can be picked up and placed in fixative, but are more easily removed if first immersed in fixative while left on the plate, allowing the gel to float off.

    The gel can be stained with Coomassie blue or silver (UNIT 6.6), or proteins can be electroeluted, electroblotted onto a polyvinylidene difluoride (PVDF) membrane for subsequent staining or sequence analysis, or transferred to a membrane for immunoblotting (UNIT 6.2). If the proteins are radiolabeled, they can be detected by autoradiography (UNIT 6.3).

ALTERNATE PROTOCOL 1

ELECTROPHORESIS IN TRIS-TRICINE BUFFER SYSTEMS

Separation of peptides and proteins under 10 to 15 kDa is not possible in the traditional Laemmli discontinuous gel system (see Basic Protocol 1). This is due to the comigration of SDS and smaller proteins, obscuring the resolution. Two approaches to obtain the separation of small proteins and peptides in the range of 5 to 20 kDa are presented: the following Tris-tricine method and a system using increased buffer concentrations (see Alternate Protocol 2). The Tris-tricine system uses a modified buffer to separate the SDS and peptides, thus improving resolution. Several precast gels are available for use with the tricine formulations (Table 6.1.4).

Table 6.1.4 Precast Gels Available from Selected Suppliers

	Format		Application				Instrument Compatibility			
	Large	Mini	2-D[a]	Native	Peptide	SDS	Bio-Rad	Cambrex	Hoefer	Invitrogen
Bio-Rad	X	X	X	X	X	X	X			
Cambrex		X	X	X	X	X	X	X	X	X
Jule	X	X	X	X	X	X	X	X	X	X
Invitrogen		X	X	X	X	X	X	X	X	X

[a]Two-dimensional analysis.

Table 6.1.5 Recipes for Tricine Peptide Separating and Stacking Gels[a]

SEPARATING AND STACKING GELS

Stock solution[b]	Separating gel	Stacking gel
30% (w/v) acrylamide/0.8% (w/v) bisacrylamide	9.80 ml	1.62 ml
Tris·Cl/SDS, pH 8.45	10.00 ml	3.10 ml
H_2O	7.03 ml	7.78 ml
Glycerol	4.00 g (3.17 ml)	—
10% (w/v) ammonium persulfate[c]	50 µl	25 µl
TEMED	10 µl	5 µl

Prepare separating and stacking gel solutions separately.

In a 50-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (Table 6.1.1), Tris·Cl/SDS, pH 8.45 (see reagents, below), and H_2O. Add glycerol to separating gel only. Degas under vacuum 10 to 15 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix; use immediately.

ADDITIONAL REAGENTS USED IN GELS

Tris·Cl/SDS, pH 8.45 *(3.0 M Tris·Cl containing 0.3% w/v SDS)*

Dissolve 182 g Tris base in 300 ml H_2O. Adjust to pH 8.45 with 1 N HCl. Add H_2O to 500 ml total volume. Filter the solution through a 0.45-µm filter, add 1.5 g SDS, and store at 4°C up to 1 month.

[a]The recipes produce 30 ml of separating gel and 12.5 ml of stacking gel, which are adequate for two gels of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the Tris-tricine buffer system of Schagger and von Jagow (1987).
[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

Additional Materials *(also see Basic Protocol 1)*

Separating and stacking gel solutions (Table 6.1.5)
2× tricine sample buffer (see recipe)
Peptide molecular weight standards (Table 6.1.6)
Cathode buffer (see recipe)
Anode buffer (see recipe)
Coomassie blue G-250 staining solution (see recipe)
10% (v/v) acetic acid
50-ml Erlenmeyer side-arm flasks

Table 6.1.6 Molecular Weights of Peptide Standards for Polyacrylamide Gel Electrophoresis[a]

Peptide	Molecular weight (Da)
Myoglobin (polypeptide backbone)	16,950
Myoglobin 1-131	14,440
Myoglobin 56-153	10,600
Myoglobin 56-131	8,160
Myoglobin 1-55	6,210
Glucagon	3,480
Myoglobin 132-153	2,510

[a]Peptide standards are commercially available from Sigma-Aldrich. See Sigma-Aldrich Technical Bulletin MWSDS70-L for molecular weight markers for proteins.

1. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 1 to 11) using the recipes in Table 6.1.5.
2. Prepare the sample (see Basic Protocol 1, step 12), but substitute 2× tricine sample buffer for the 2× SDS sample buffer, and heat the sample at 40°C for 30 to 60 min to improve solubilization prior to loading. Use peptide molecular weight standards (Table 6.1.6).

   If proteolytic activity is a problem (see Basic Protocol 1, step 12), heating samples to 100°C for 3 to 5 min may be required.
3. Set up the electrophoresis apparatus and load the gel (see Basic Protocol 1, steps 13 to 20), but use cathode buffer or water to rinse the wells, use cathode buffer in the upper buffer chamber, and use anode buffer in the lower buffer chamber.

   The cathode buffer contains the tricine.
4. Connect the power supply to the cell and run 1 hr at 30 V (constant voltage) followed by 4 to 5 hr at 150 V (constant voltage). Use a heat exchanger to keep the electrophoresis chamber at room temperature.
5. After the tracking dye has reached the bottom of the separating gel, disconnect the power supply.

   Refer to Safety Considerations under Electricity and Electrophoresis.

   Coomassie blue G-250 is used as a tracking dye instead of bromphenol blue because it moves ahead of the smallest peptides.
6. Disassemble the gel (see Basic Protocol 1, steps 23 to 26). Stain proteins in the gel for 1 to 2 hr in Coomassie blue G-250 staining solution. Follow by destaining with 10% acetic acid, changing the solution every 30 min until background is clear (3 to 5 changes). For higher sensitivity, use silver staining as a recommended alternative.

   Prolonged staining and destaining will result in the loss of resolution of the smaller proteins (<10 kDa). Proteins diffuse within the gel and out of the gel, resulting in a loss of staining intensity and resolution.

ALTERNATE PROTOCOL 2

NONUREA PEPTIDE SEPARATIONS WITH TRIS BUFFERS

A simple modification of the traditional Laemmli buffer system presented in Basic Protocol 1, in which the increased concentration of buffers provides better separation between the stacked peptides and the SDS micelles, permits reasonable separation of peptides as small as 5 kDa.

Additional Materials *(also see Basic Protocol 1)*

Separating and stacking gel solutions (Table 6.1.7)
2× Tris·Cl/SDS, pH 8.8 (dilute 4× Tris·Cl/SDS, pH 8.8; Table 6.1.1)
2× SDS electrophoresis buffer (see recipe)

1. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 1 to 11), using the modified recipes in Table 6.1.7. After removing the isobutyl alcohol overlay from the separating gel, rinse with 2× Tris·Cl/SDS, pH 8.8, rather than 1× Tris·Cl/SDS.

2. Prepare the sample and load the gel (see Basic Protocol 1, steps 12 to 20), but substitute 2× SDS electrophoresis buffer for the 1× SDS electrophoresis buffer.

Table 6.1.6 lists the standards for small protein separations.

Table 6.1.7 Recipes for Modified Laemmli Peptide Separating and Stacking Gels[a]

SEPARATING AND STACKING GELS

Stock solution[b]	Separating gel	Stacking gel
30% (w/v) acrylamide/0.8% (w/v) bisacrylamide	10.00 ml	0.65 ml
8× Tris·Cl, pH 8.8	3.75 ml	—
4× Tris·Cl, pH 6.8	—	1.25 ml
10% (w/v) SDS[c]	0.15 ml	50 µl
H_2O	1.00 ml	3.00 ml
10% (w/v) ammonium persulfate[c]	50 µl	25 µl
TEMED	10 µl	5 µl

Prepare separating and stacking gel solutions separately.

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 8× Tris·Cl, pH 8.8, or 4× Tris·Cl, pH 6.8 (see reagents below), 10% SDS, and H_2O. Degas under vacuum 10 to 15 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

ADDITIONAL REAGENTS USED IN GELS

4× Tris·Cl, pH 6.8 *(0.5 M Tris·Cl)*

Dissolve 6.05 g Tris base in 40 ml H_2O. Adjust to pH 6.8 with 1 N HCl. Add H_2O to 100 ml total volume. Filter the solution through a 0.45-µm filter and store up to 1 month at 4°C.

8× Tris·Cl, pH 8.8 *(3.0 M Tris·Cl)*

Dissolve 182 g Tris base in 300 ml H_2O. Adjust to pH 8.8 with 1 N HCl. Add H_2O to 500 ml total volume. Filter the solution through a 0.45-µm filter and store up to 1 month at 4°C.

[a]The recipes produce 15 ml of separating gel and 5 ml of stacking gel, which are adequate for one gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the modified Laemmli peptide separation system of Okajima et al. (1993).
[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

3. Run the gel (see Basic Protocol 1, steps 21 and 22).

   Note that the separations will take ~25% longer than those using Basic Protocol 1. The increased buffer concentrations lead to faster transit through the stacking gel but lower mobility in the resolving gel.

4. Disassemble the gel (see Basic Protocol 1, steps 23 to 26).

   Proteins in the gel may now be stained.

ALTERNATE PROTOCOL 3

CONTINUOUS SDS-PAGE

With continuous SDS-PAGE, the same buffer is used for both the gel and electrode solutions. Although continuous gels lack the resolution of the discontinuous systems, they are extremely versatile, less prone to mobility artifacts, and much easier to prepare. The stacking gel is omitted.

Additional Materials *(also see Basic Protocol 1)*

Separating gel solution (Table 6.1.8)
2× and 1× phosphate/SDS sample buffer (see recipe)
1× phosphate/SDS electrophoresis buffer (see recipe)

Table 6.1.8 Recipes for Separating Gels for Continuous SDS-PAGE[a]

SEPARATING GEL

	Final acrylamide concentration in the separating gel (%)[c]										
Stock solution[b]	5	6	7	8	9	10	11	12	13	14	15
30% (w/v) acrylamide/0.8% (w/v) bisacrylamide	2.5	3.00	3.50	4.00	4.50	5.00	5.50	6.00	6.50	7.00	7.50
4× phosphate/SDS, pH 7.2	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.25	6.75	6.25	5.75	5.25	4.75	4.25	3.75
10% (w/v) ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Preparation of separating gel

In a 25-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× phosphate/SDS, pH 7.2, and H_2O. Degas under vacuum about 5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

ADDITIONAL REAGENTS USED IN GELS

4× phosphate/SDS, pH 7.2 *(0.4 M sodium phosphate/0.4% w/v SDS)*

Mix 46.8 g $NaH_2PO_4 \cdot H_2O$, 231.6 g $Na_2HPO_4 \cdot 7H_2O$, and 12 g SDS in 3 liters H_2O. Store at 4°C for up to 3 months.

[a] The recipes produce 15 ml of separating gel, which is adequate for one gel of dimensions 0.75 mm × 14 cm × 14 cm. The recipes are based on the original continuous phosphate buffer system of Weber et al. (1972).
[b] All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c] Volumes are in milliliters. The desired percentage of acrylamide in the separating gel depends on the molecular size of the protein being separated. See Basic Protocol 1, annotation to step 3.
[d] Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

1. Prepare and pour a single separating gel (see Basic Protocol 1, steps 1 to 4), except use the recipe in Table 6.1.8 and fill the gel sandwich to the top. Omit the stacking gel. Insert the comb (see Basic Protocol 1, step 10) and allow the gel to polymerize 30 to 60 min at room temperature.

2. Mix the protein sample 1:1 with 2× phosphate/SDS sample buffer and heat to 100°C for 2 min.

   For large sample volumes or samples suspended in high-ionic-strength buffers (>50 mM), dialyze against 1× sample buffer prior to electrophoresis. Note that the precautions about proteases (see Basic Protocol 1, step 12) apply here.

3. Assemble the electrophoresis apparatus and load the sample (see Basic Protocol 1, steps 13 to 20) using the phosphate/SDS electrophoresis buffer. Load empty wells with 1× phosphate/SDS sample buffer.

4. Connect the power supply and start the run with 15 mA per 0.75-mm-thick gel until the tracking dye has entered the gel. Continue electrophoresis at 30 mA for 3 hr (5% gel), 5 hr (10% gel), 8 hr (15% gel), or until the dye reaches the bottom of the gel.

   Use temperature control if available to maintain the gel at 15° to 20°C. SDS will precipitate below 15°C in this system.

5. Disassemble the gel (see Basic Protocol 1, steps 23 to 26).

   See Safety Considerations in introduction. Proteins in the gel may now be stained.

CASTING AND RUNNING ULTRATHIN GELS

ALTERNATE PROTOCOL 4

Ultrathin gels provide superb resolution but are difficult to handle. In this application, gels are cast on GelBond, a Mylar support material. Silver staining is recommended for the best resolution. Combs and spacers for gels <0.5 mm thick are not readily available for most protein electrophoresis units. However, by adapting combs and spacers used for DNA sequencing, casting gels from 0.2 to 0.5 mm thick is straightforward.

Additional Materials *(also see Basic Protocol 1)*

95% (v/v) ethanol
GelBond (Lonza) cut to a size slightly smaller than the gel plate dimensions
Glue stick
Ink roller (available from art supply stores)
Combs and spacers (0.19 to 0.5 mm; sequencing gel spacers and combs can be cut to fit)

1. Wash gel plates with water-based laboratory detergent followed by successive rinses with hot tap water, deionized water, and finally 95% ethanol. Allow to air dry.

   Gel plates must be extremely clean for casting thin gels. Gloves are needed throughout these procedures to prevent contamination by proteins on the surface of skin.

2. Apply a streak of adhesive from a glue stick to the bottom edge of the glass plate. Quickly position the GelBond with the hydrophobic side down (a drop of water will bead up on the hydrophobic surface). Apply pressure with Kimwipe tissue to attach the GelBond firmly to the plate. Finally, pull the top portion of the GelBond back, place a few drops of water underneath, and roll flat with an ink roller.

   Make sure the GelBond does not extend beyond the edges of the upper and lower sealing surface of the plate. This will cause it to buckle on sealing. Reposition the GelBond if needed to prevent it from extending beyond the glass plate. Material may also be trimmed to fit flush with the plate edge.

3. Assemble the gel cassette according to the manufacturer's instructions (also see Basic Protocol 1, steps 1 and 2). Just prior to assembly, blow air over the surface of both the GelBond and the opposing glass surface to remove any particulate material (e.g., dust).

   Sequencing gel spacers can be easily adapted. First, cut the spacers slightly longer than the length of the gel plate. Position a spacer along each edge of the glass plate and assemble the gel sandwich, clamping in place. With a razor blade, trim the excess spacer at top and bottom to get a reusable spacer exactly the size of the plate.

4. Prepare and pour the separating and stacking gels (see Basic Protocol 1, steps 3 to 9). In place of the Teflon comb, insert a square well sequencing comb cut to fit within the gel sandwich. Allow the stacking gel to polymerize 30 to 45 min at room temperature.

   Less solution is needed for ultrathin gels. For example, a 0.5-mm-thick gel requires 33% less gel solution than a 0.75-mm gel.

5. Prepare the sample and load the gel (see Basic Protocol 1, steps 12 to 20).

   When preparing protein samples for ultrathin gels, 3 to 4 μl at 5 μg protein/μl is required for Coomassie blue R-250 staining, whereas 10-fold less is needed for silver staining.

6. Run the gel (see Basic Protocol 1, steps 21 and 22), except conduct the electrophoresis at 7 mA/gel (0.25-mm-thick gels) or 14 mA/gel (0.5-mm-thick gels) for 4 to 5 hr.

7. When the separation is complete, disassemble the unit and remove the gel (see Basic Protocol 1, steps 23 to 26) with the GelBond still attached. With a gloved hand, wash away the adhesive material from the back of the GelBond under a stream of water before proceeding to protein detection.

   Either Coomassie blue or silver staining may be used, but silver staining produces particularly fine resolution with thin GelBond-backed gels. Compared to staining thicker (>0.75 mm) gels, thin (<0.75 mm) gels stain and destain more quickly. Although the optimum staining times must be empirically determined, all steps in Coomassie blue and silver staining procedures are generally reduced by half.

SUPPORT PROTOCOL 1

CASTING MULTIPLE SINGLE-CONCENTRATION GELS

Casting multiple gels at one time has several advantages. All the gels are identical, so sample separation is not affected by gel-to-gel variation. Furthermore, casting ten gels is only slightly more difficult than casting two gels. Once cast, gels can be stored for several days in a refrigerator.

Additional Materials *(also see Basic Protocol 1)*

Separating and stacking gels for single-concentration gels (Table 6.1.9)
Multiple gel caster (Bio-Rad, Hoefer)
100-ml disposable syringe and flat-tipped needle
Extra plates and spacers
14 × 14–cm acrylic blocks or polycarbonate sheets
250- and 500-ml side-arm flasks (used in gel preparation)
Long razor blade *or* plastic wedge (Wonder Wedge, Hoefer)
Resealable plastic bags

Pour the separating gel

1. Assemble the multiple gel caster according to the manufacturer's instructions.

   With the Hoefer unit, make sure to insert the large triangular space filler plug in the base of the caster. The plug is removed when casting gradient gels (see Support Protocol 2).

Table 6.1.9 Recipes for Multiple Single-Concentration Polyacrylamide Gels[a]

SEPARATING GEL

Stock solution[b]	Final acrylamide concentration in the separating gel (%)[c]										
	5	6	7	8	9	10	11	12	13	14	15
30% (w/v) acrylamide/ 0.8% (w/v) bisacrylamide	52	62	72	83	93	103	114	124	134	145	155
4× Tris·Cl/SDS, pH 8.8	78	78	78	78	78	78	78	78	78	78	78
H_2O	181	171	160	150	140	129	119	109	98	88	78
10% (w/v) ammonium persulfate[d]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
TEMED	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21

Preparation of separating gel

In a 500-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× Tris·Cl/SDS, pH 8.8 (Table 6.1.1), and H_2O. Degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

STACKING GEL

In a 250-ml side-arm flask, mix 13.0 ml 30% acrylamide/0.8% bisacrylamide solution, 25 ml 4× Tris·Cl/SDS, pH 6.8 (Table 6.1.1), and 61 ml H_2O. Degas under vacuum ~5 min. Add 0.25 ml 10% ammonium persulfate and 50 µl TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce about 300 ml of separating gel and 100 ml of stacking gel, which are adequate for ten gels of dimensions 1.5 mm × 14 cm × 14 cm, with extra solution should there be a leak or spill while casting the gels. For thinner spacers or fewer gels, calculate volumes using the equation in the annotation to step 4. The recipes are based on the SDS (denaturing) discontinuous buffer system of Laemmli (1970).
[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c]Volumes in table body are in milliliters. The desired percentage of acrylamide in separating gel depends on the molecular size of the protein being separated. See Basic Protocol 1, annotation to step 3.
[d]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the persulfate, TEMED, or both.

2. Assemble glass sandwiches and stack them in the casting chamber. Stack up to ten 1.5-mm gels and fill in extra space with acrylic blocks or polycarbonate sheets to hold the sandwiches tightly in place. Make sure the spacers are straight along the top, right, and left edges of the glass plates and that all edges of the stack are flush.

   The presence of loosely fitting sandwiches in the caster will lead to unevenly cast gels, creating distortions during electrophoresis. Polycarbonate inhibits gel polymerization. Therefore, if polycarbonate sheets are placed in the caster before and after the set of glass sandwiches, the entire set will slide out as one block after polymerization. Placing polycarbonate sheets between each gel sandwich makes them easier to separate from one another after polymerization.

3. Place the front sealing plate on the casting chamber, making sure the stack fits snugly. Secure the plate with four spring clamps and tighten the bottom thumb screws.

4. Prepare the separating (resolving) gel solution (Table 6.1.9).

   A 12-cm separating gel with a 4-cm stacking gel is recommended.

   If fewer than ten gels are prepared (Table 6.1.9), use the following formula to estimate the amount of separating gel volume needed:

   volume = gel no. × height (cm) × width (cm) × thickness (cm) + 4 × gel no. + 10 ml.

5. Using a 100-ml disposable syringe with flat-tipped needle, inject the resolving gel solution down the side of one spacer into the multiple caster. A channel in the silicone plug distributes the solution throughout the whole caster. Avoid introducing bubbles by giving the caster a quick tap on the benchtop once the caster is filled.
6. Overlay the center of each gel with 100 μl H_2O-saturated isobutyl alcohol and let polymerize for 1 to 2 hr.
7. Drain off the overlay and rinse the surface with 1× Tris·Cl/SDS, pH 8.8. If the gels will not be used immediately, skip to step 12.

Pour the stacking gel

8. Prepare the stacking gel solution either singly (see Basic Protocol 1, step 8) or for all the gels at once (Table 6.1.9).

   The stacking gel solution should be prepared just before it is poured.

9. Fill each sandwich in the caster with stacking gel solution.
10. Insert a comb into each sandwich and let the gel polymerize for 2 hr.

    Insert the combs at a 45° angle to avoid trapping air underneath the comb teeth. Air bubbles will inhibit polymerization and cause dents in the wells and a distorted pattern of protein bands.

11. Remove the combs and rinse wells with 1× SDS electrophoresis buffer.

Remove the gels from the caster

12. Remove the gels from the caster and separate by carefully inserting a long razor blade or knife between the gel sandwiches.

    A plastic wedge (Hoefer's Wonder Wedge) also works well.

13. Clean the outside of each gel plate with running water to remove the residual polymerized and unpolymerized acrylamide.
14. Overlay gels to be stored with 1× Tris·Cl/SDS, pH 8.8, place in a resealable plastic bag, and store at 4°C until needed (up to 1 week).

ALTERNATE PROTOCOL 5

SEPARATION OF PROTEINS ON GRADIENT GELS

Gels that consist of a gradient of increasing polyacrylamide concentration resolve a much wider size range of proteins than standard single-concentration gels (see Critical Parameters and Troubleshooting). The protein bands are also much sharper, particularly in the low-molecular-weight range. Unlike single-concentration gels, gradient gels separate proteins in a way that can be represented easily to give a linear plot from 10 to 200 kDa. This facilitates molecular weight estimations.

The quantities given below provide separating gel solution sufficient for two 0.75-mm gels (~7 ml of each concentration) or one 1.5-mm gel (~14 ml of each concentration). Volumes can be adjusted to accommodate gel sandwiches of different dimensions.

Additional Materials *(also see Basic Protocol 1)*

Light and heavy acrylamide gel solutions (Table 6.1.10)
Bromphenol blue (optional; for checking practice gradient)
10% ammonium persulfate (prepare fresh)
TEMED

Gradient maker (30 to 50 ml, Hoefer SG30 or SG50; *or* 30 to 100 ml, Bio-Rad 385)
Tygon tubing with micropipet tip
Peristaltic pump (optional; e.g., Markson A-13002, A-34040, or A-34105 minipump)
Whatman 3MM filter paper

Set up the gradient maker and prepare the gel solutions

1. Assemble the magnetic stirrer and gradient maker on a ring stand as shown in Figure 6.1.2. Connect the outlet valve of the gradient maker to Tygon tubing attached to a micropipet tip that is placed over the vertical gel sandwich. If desired, place a peristaltic pump in line between the gradient maker and the gel sandwich.

   A peristaltic pump will simplify casting by providing a smooth flow rate.

2. Place a small stir-bar into the mixing chamber of the gradient maker (i.e., the chamber connected to the outlet).

Table 6.1.10 Light and Heavy Acrylamide Gel Solutions for Gradient Gels

	Acrylamide concentration in light gel solution (%)[a,b]									
Stock solution	5	6	7	8	9	10	11	12	13	14
30% acrylamide/0.8% bisacrylamide[c]	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0
4× Tris·Cl/SDS, pH 8.8[c]	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	8.75	8.25	7.75	7.25	6.75	6.25	5.75	5.25	4.75	4.25
10% ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED[d]	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005

	Acrylamide concentration in heavy gel solution (%)[a,b]										
Stock solution	10	11	12	13	14	15	16	17	18	19	20
30% acrylamide/0.8% bisacrylamide[c]	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
4× Tris·Cl/SDS, pH 8.8[c]	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0
Sucrose (g)	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25
10% ammonium persulfate[d]	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED[d]	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005

[a] To survey proteins ≥10 kDa, 5% to 20% gradient gels are recommended. To expand the range between 10 and 200 kDa, a 10% to 20% gradient gel is recommended.
[b] Volumes are in milliliters (sucrose is in grams). Keep light gel solution at room temperature prior to use (no longer than 1 hr). Keep heavy solution on ice.
[c] See Table 6.1.1 for preparation.
[d] Ammonium persulfate and TEMED are added directly to the gradient chambers immediately before the gel is poured. It is best to prepare ammonium persulfate fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

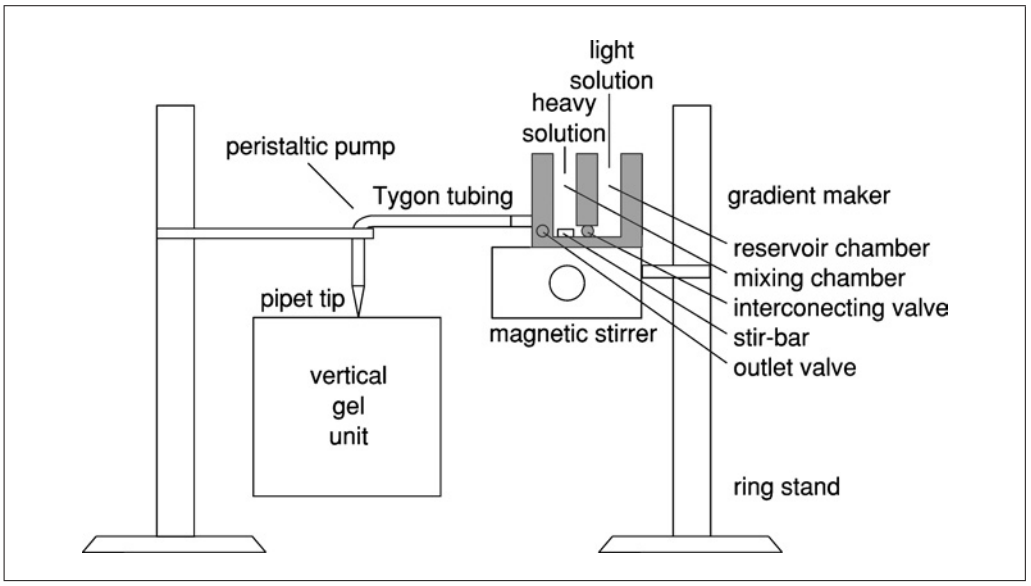

Figure 6.1.2 Gradient gel setup. A peristaltic pump, though not required, will provide better control.

3. Using the recipes in Table 6.1.10, prepare light and heavy acrylamide gel solutions *without* ammonium persulfate or TEMED.

   Recommended gradient ranges are 5% to 20% for most applications (to separate proteins of 10 to several hundred kilodaltons).

   Deaeration is not recommended for either the light or heavy solution. Omitting the deaeration will allow polymerization to proceed more slowly, letting the gradient establish itself in the gel sandwich before polymerization takes place.

   Keep the heavy solution on ice until use. Once the ammonium persulfate is added to the heavy solution, it will polymerize without TEMED, albeit more slowly; keeping the solution on ice prevents this. The gel solution will come to room temperature during casting. The higher the percentage of acrylamide, the more severe the problem of premature polymerization.

4. With the outlet port and interconnecting valve between the two chambers closed, pipet 7 ml of light (low-concentration) acrylamide gel solution into the reservoir chamber for one 0.75-mm-thick gradient gel.

   A practice run with heavy and light solutions is recommended. Bromphenol blue should be added to the heavy solution to demonstrate linearity of the practice gradient.

5. Open the interconnecting valve briefly to allow a small amount (~200 µl) of light solution to flow through the valve and into the mixing chamber.

   The presence of air bubbles in the interconnecting valve may obstruct the flow between chambers during casting.

6. Add 7 ml of heavy (high-concentration) acrylamide gel solution to the mixing chamber.

7. Add 23 µl of 10% ammonium persulfate and ~2.3 µl TEMED per 7 ml acrylamide solution to each chamber. Mix the solutions in each chamber with a disposable pipet.

Form the gradient and cast the gel

8. Open the interconnecting valve completely.

   Some of the heavy solution will flow back into the reservoir chamber containing light solution as the two chambers equilibrate. This will not affect the formation of the gradient.

9. Turn on the magnetic stirrer and adjust the rate to produce a slight vortex in the mixing chamber.

10. Open the outlet of the gradient maker slowly. Adjust the outlet valve to a flow rate of 2 ml/min. If using a peristaltic pump, calibrate the flow rate with a graduated cylinder prior to casting the gel.

    Some adjustment of the flow rate may be necessary during casting. If the light solution is not flowing into the mixing chamber, a bubble may be caught in the interconnecting valve. Quickly close the outlet and cover the top of the reservoir chamber with a gloved thumb. Push down with the thumb to increase the pressure in the chamber and force the air bubble out of the center valve.

11. Fill the gel sandwich from the top. Place the pipet tip against one side of the sandwich so the solution flows down one plate only. The heavy solution will flow into the sandwich first, followed by progressively lighter solution.

12. Watch as the last of the light solution drains into the outlet tube and adjust the flow rate to ensure that the last few milliliters of solution do not flow quickly into the gel sandwich and disturb the gradient.

13. Overlay the gradient gel with H_2O-saturated isobutyl alcohol. Allow the gel to polymerize ~1 hr.

    In this gel system, the gel will polymerize from the bottom (i.e., heavy solution) up. Because polymerization is an exothermic reaction, heat can be felt evolving from the bottom of the gel sandwich during polymerization. A sharp optical discontinuity at the gel-overlay interface indicates that polymerization has occurred. In general, 1 hr is adequate for polymerization.

14. Remove the H_2O-saturated isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8. Cast the stacking gel (see Basic Protocol 1, steps 8 to 11).

    The gel can be covered with 1× Tris·Cl/SDS, pH 8.8, sealed in a plastic bag, and stored for up to 1 week.

Load and run the gel

15. Prepare the protein sample and protein molecular-weight-standards mixture. Load and run the gel (see Basic Protocol 1, steps 13 to 26).

    The gel can be stained with Coomassie blue or silver (UNIT 6.6).

16. After staining, dry the gels onto Whatman 3MM or equivalent filter paper.

    Gradient gels >0.75 mm thick require special handling during drying to prevent cracking. The simplest approach to drying gradient gels is to use thin gels; ≤0.75-mm gradient gels with ≤20% acrylamide solutions will dry without cracking as long as the vacuum pump is working properly and the cold trap is dry at the onset of drying. For gradient gels >0.75 mm thick, add 3% (w/v) glycerol to the final destaining solution to help prevent cracking. Another method is to dehydrate and shrink the gel in 30% methanol for up to 3 hr prior to drying. Then place the gel in distilled water for 5 min before drying.

CASTING MULTIPLE GRADIENT GELS

SUPPORT PROTOCOL 2

Casting gradient gels in a multiple gel caster has several advantages. In addition to the time savings, batch casting produces gels that are essentially identical. This is particularly important for gradient gels, where slight variations in casting technique can cause variations in protein mobility. The gels may be stored for up to 1 week after casting to ensure internal consistency from run to run during the week. Furthermore, gels with several ranges of concentrations (e.g., 5% to 20% and 10% to 20% acrylamide) can be cast and stored, giving much more flexibility to optimize separations.

Additional Materials *(also see Alternate Protocol 5)*

Plug solution (see recipe)
Light and heavy acrylamide gel solutions for multiple gradient gels (Table 6.1.11)
TEMED
H_2O-saturated isobutyl alcohol

Multiple gel caster (Bio-Rad, Hoefer)
Peristaltic pump (25 ml/min)
500- or 1000-ml gradient maker (Bio-Rad, Hoefer)
Tygon tubing

Set up system and pour separating gel

1. Assemble the multiple caster as in casting multiple single-concentration gels (see Support Protocol 1, steps 1 to 3), making sure to remove the triangular space filler plugs in the bottom of the caster.

   The plug is used only when casting single-concentration gels.

Table 6.1.11 Light and Heavy Acrylamide Gel Solutions for Casting Multiple Gradient Gels

	Acrylamide concentration in light gel solution (%)[a,b]									
Stock solution	5	6	7	8	9	10	11	12	13	14
30% acrylamide/0.8% bisacrylamide[c]	28	33	39	44	50	55	61	66	72	77
4× Tris·Cl/SDS, pH 8.8[c]	41	41	41	41	41	41	41	41	41	41
H_2O	96	91	85	80	74	69	63	58	52	47
10% ammonium persulfate[d]	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
TEMED	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054

	Acrylamide concentration in heavy gel solution (%)[a,b]										
Stock solution	10	11	12	13	14	15	16	17	18	19	20
30% acrylamide/0.8% bisacrylamide[c]	55	61	66	72	77	83	88	94	99	105	110
4× Tris·Cl/SDS, pH 8.8[c]	41	41	41	41	41	41	41	41	41	41	41
H_2O	55	50	44	39	33	28	22	17	11	5.5	0
Sucrose (g)	25	25	25	25	25	25	25	25	25	25	25
10% ammonium persulfate[d]	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55	0.55
TEMED	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054

[a]To survey proteins ≥10 kDa, 5% to 20% gradient gels are recommended. To expand the range between 10 and 200 kDa, a 10% to 20% gradient gel is recommended.
[b]Volumes are in milliliters (sucrose is in grams). Recipes produce ten 1.5-mm-thick gradient gels with 10 ml extra solution to account for losses in tubing. Keep light gel solution at room temperature prior to use (no longer than 1 hr). Keep heavy solution on ice.
[c]See Table 6.1.1 for preparation.
[d]Best to prepare fresh. Failure to form a firm gel usually indicates a problem with the ammonium persulfate, TEMED, or both.

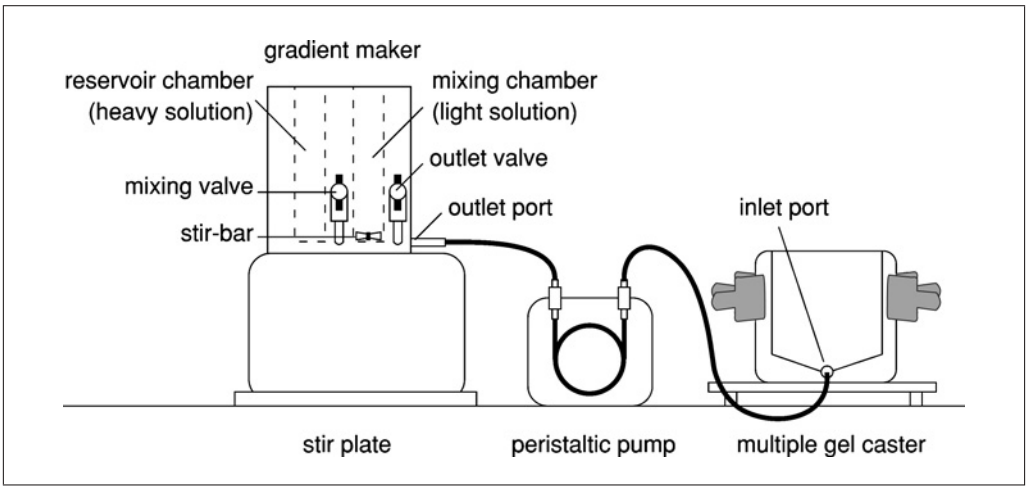

Figure 6.1.3 Setup for casting multiple gradient gels. Casting multiple gradient gels requires a peristaltic pump and a multiple gel caster. Gel solution is introduced through the bottom of the multiple caster.

2. Set up the peristaltic pump (Fig. 6.1.3). Using a graduated cylinder and water, adjust the flow rate so that the volume of the gradient solution plus volume of plug solution is poured in ~15 to 18 min (~25 ml/min).

3. Set up the gradient maker. Close all valves and place a stir-bar in the mixing chamber, which is the one with the outlet port. Attach one end of a piece of Tygon tubing to the outlet of the gradient maker. Run the other end of the tubing through the peristaltic pump and attach it to the red inlet port at the bottom of the caster.

   Choose a gradient maker that holds no more than four times the total volume of the gradient solution to be poured (i.e., a 1000- or 500-ml gradient maker).

4. Prepare solutions for the gradient maker (Table 6.1.11).

   Deaeration is not recommended for gradient gels.

5. *Immediately* after adding TEMED to the gel solutions, pour the light (low-concentration) solution into the mixing chamber (the one with the port). Open the mixing valve slightly to allow the tunnel to fill and to avoid air bubbles. Close the valve again and pour the heavy (high-concentration) acrylamide solution into the reservoir chamber.

6. Start the magnetic stirrer and open the outlet valve; then start the pump and open the mixing valve.

   In units for casting multiple gels, acrylamide solution flows in from the bottom. To use a multiple casting unit, the light solution is placed in the mixing chamber and the heavy solution in the reservoir. This is the reverse of casting a single gel (see Alternate Protocol 5). Thus, light solution enters the multiple caster first, followed by progressively heavier solution. Finally, the acrylamide solution is stabilized in the multiple caster with a heavy plug solution and allowed to polymerize (see step 8 and manufacturer's instructions).

7. When almost all the acrylamide solution is gone from the gradient maker, stop the pump and close the mixing valve. Tilt the gradient maker toward the outlet side and remove the last milliliters of the mix. Do not allow air bubbles to enter the tubing.

8. Add the plug solution to the mixing chamber and start the pump. Make sure that no bubbles are introduced. Continue pumping until the bottom of the caster is filled with plug solution to just below the glass plates; then turn off the pump. Clamp the tubing close to the red port of the casting chamber.

9. Quickly overlay each separate gel sandwich with 100 µl H_2O-saturated isobutyl alcohol. Use the same amount on each sandwich. Allow the gels to polymerize for ~1 hr.

   Failure to use the same amount of overlay solution will cause the gel sandwiches to polymerize at different heights.

10. Drain off the overlay and rinse the surface of the gels with 1× Tris·Cl/SDS, pH 8.8.

Pour stacking gel and remove gels from caster

11. Prepare and cast the stacking gel as in casting multiple single-concentration gels (see Support Protocol 1, steps 8 to 11).

12. Remove gels from the caster and clean the gel sandwiches (see Support Protocol 1, steps 12 and 13). Store gels, if necessary, according to the instructions for multiple single-concentration gels (see Support Protocol 1, step 14).

BASIC PROTOCOL 2

ELECTROPHORESIS IN SINGLE-CONCENTRATION MINIGELS

Separation of proteins in a small-gel format is becoming increasingly popular for applications that range from isolating material for peptide sequencing to performing routine protein separations. The unique combination of speed and high resolution is the foremost advantage of small gels. Additionally, small gels are easily adapted to single-concentration, gradient, and two-dimensional SDS-PAGE procedures. The minigel procedures described are adaptations of larger gel systems.

This protocol describes the use of a multiple gel caster. The caster is simple to use, and up to five gels can be prepared at one time with this procedure. Single gels can be prepared using adaptations in the manufacturer's instructions. A multiple gel caster is the only practical way to produce small linear polyacrylamide gradient gels (see Support Protocol 3).

Materials

Minigel vertical gel unit (Hoefer Mighty Small SE 250/280 *or* Bio-Rad Mini-Protean II) with glass plates, clamps, and buffer chambers
0.75-mm spacers
Multiple gel caster (Hoefer SE-275/295 *or* Bio-Rad Mini-Protean II multicasting chamber)
Acrylic plate (Hoefer SE-217 *or* Bio-Rad 165-1957) or polycarbonate separation sheet (Hoefer SE-213 *or* Bio-Rad 165-1958)
10- and 50-ml syringes
Combs (Teflon, Hoefer SE-211A series or Bio-Rad Mini-Protean II)
Long razor blade
Micropipet
Additional reagents and equipment for standard denaturing SDS-PAGE (see Basic Protocol 1)

Pour the separating gel

1. Assemble each gel sandwich by stacking, in order, the notched (Hoefer) or small rectangular (Bio-Rad) plate, 0.75-mm spacers, and the larger rectangular plate. Be sure to align the spacers properly, with the ends flush with the top and bottom edge of the two plates, when positioning the sandwiches in the multiple gel caster (Fig. 6.1.4).

   The protocol described is basically for the Hoefer system. For other systems, make adjustments according to the manufacturer's instructions. Alternatively, precast minigels can be purchased from a number of suppliers (see Table 6.1.4).

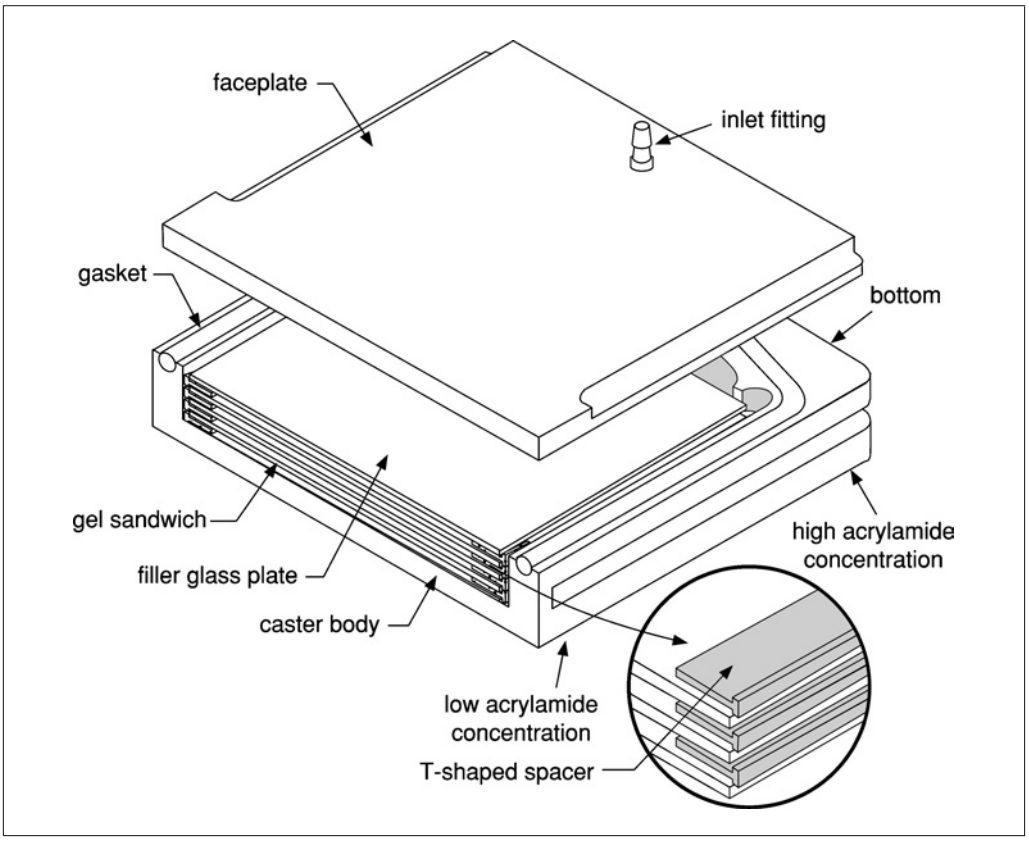

Figure 6.1.4 Minigel sandwiches positioned in the multiple gel caster. Extra glass or acrylic plates or polycarbonate sheets are used to fill any free space in the caster and to ensure that the gel sandwiches are held firmly in place.

The multiple casters from Hoefer have a notch in the base designed for casting gradient gels. A silicone rubber insert fills up this space when casting single-concentration gels. The Hoefer spacers are T-shaped to prevent slipping. The flanged edge of the spacer must be positioned against the outside edge of the glass plate. Placing a sheet of wax paper between the gel sandwiches will help separate the sandwiches after polymerization.

2. Fit the gel sandwiches tightly in the multiple gel caster. Use an acrylic plate or polycarbonate separation sheet to eliminate any slack in the chamber.

   Loosely fitting sandwiches in the caster will lead to unevenly cast gels, creating distortions during electrophoresis.

3. Place the front faceplate on the caster, clamp it in place against the silicone gasket, and verify alignment of the glass plates and spacers.

4. Prepare the separating gel solution as directed in Table 6.1.1. For five 0.75-mm-thick gels, prepare ~30 ml solution (i.e., double the volumes listed).

   To compute the total gel volume needed, multiply the area of the gel (e.g., 7.3 × 8.3 cm) by the thickness of the gel (e.g., 0.75 mm) and then by the number of gels in the caster. If needed, add ~4 to 5 ml of extra gel solution to account for the space around the outside of the gel sandwiches.

   Do not add TEMED and ammonium persulfate until just before use.

5. Fill a 50-ml syringe with the separating gel solution and slowly inject it into the caster until the gels are 6 cm high, allowing 1.5 cm for the stacking gel.

6. Overlay each gel with 100 µl H_2O-saturated isobutyl alcohol. Allow the gels to polymerize for ~1 hr.

Pour the stacking gel

7. Remove the isobutyl alcohol and rinse with 1× Tris·Cl/SDS, pH 8.8.

   Stacking gels can be cast one at a time with the gel mounted on the electrophoresis unit, or all at once in the multiple caster.

8. Practice placing a comb in the gel sandwiches before preparing the stacking gel solution. Press the comb against the rectangular or taller plate so that all teeth of the comb are aligned with the opening in the gel sandwich, then insert into the sandwich. Remove combs after practicing.

9. Prepare the stacking gel solution (2 ml per gel) as directed in Table 6.1.1. Fill a 10-ml syringe with stacking gel solution and inject the solution into each gel sandwich.

10. Insert combs, taking care not to trap bubbles. Allow the gels to polymerize 1 hr.

11. Remove the front faceplate. Carefully pull the gels out of the caster, using a long razor blade to separate the sandwiches.

    If the gels are left to polymerize for prolonged periods, they will be difficult to remove from the caster.

    The gels can be stored tightly wrapped in plastic wrap with the combs left in place inside a sealable bag to prevent drying for ∼1 week. Without the stacking gel, the separating gel can be stored for 2 to 3 weeks. Keep gels moist with 1× Tris·Cl/SDS, pH 8.8, at 4°C. Do not store gels in the multiple caster.

Prepare the sample, load the gel, and conduct electrophoresis

12. Remove the combs and rinse the sample wells with 1× SDS electrophoresis buffer. Place a line indicating the bottom of each well on the front glass plate with a marker.

13. Fill the upper and lower buffer chambers with 1× SDS electrophoresis buffer. The upper chamber should be filled to 1 to 2 cm over the notched plate.

14. Prepare the protein sample and protein standards mixture (see Basic Protocol 1, step 12).

15. Load the sample using a micropipet. Insert the pipet tip through the upper buffer and into the well. The mark on the glass plate will act as a guide. Dispense the sample into the well.

    For a complex mixture, 20 to 25 μg protein in 10 μl SDS sample buffer will give a strongly stained Coomassie blue pattern. Much smaller amounts (1 to 5 μg) are required for highly purified proteins, and a 10- to 100-fold smaller amount of protein in the same volume (e.g., 10 μl) is required for silver staining.

16. Electrophorese samples at 10 to 15 mA per 0.75-mm gel until the dye front reaches the bottom of the gel (∼1 to 1.5 hr).

17. Disassemble the gel (see Basic Protocol 1, steps 23 to 26). Proceed with detection of proteins.

SUPPORT PROTOCOL 3

PREPARING MULTIPLE GRADIENT MINIGELS

Polyacrylamide gradients not only enhance the resolution of larger-format gels but also greatly improve protein separation in the small format. Casting gradient minigels one at a time is not generally feasible because of the small volumes used, but multiple gel casters make it easy to cast several small gradient gels at one time. The gels are cast from the bottom in multiple casters, with the light acrylamide solution entering first. This is the opposite of casting one gel at a time, in which the heavy solution enters from the top of the gel sandwich and flows down to the bottom.

Additional Materials *(also see Basic Protocol 2)*

Plug solution (see recipe)

Additional reagents and equipment for preparing gradient gels (see Alternate Protocol 5)

Set up the system and prepare the gel solutions

1. Assemble minigel sandwiches in the multiple gel caster as described for single-concentration minigels (see Basic Protocol 2, steps 1 to 3).

2. Set up the 30-ml gradient maker, magnetic stirrer, peristaltic pump (optional), and Tygon tubing as in Figure 6.1.3. Connect the outlet of the 30-ml gradient maker to the inlet at the base of the front faceplate of the caster.

   The monomer solution will be introduced through the inlet at the bottom of the front faceplate of the caster first, followed by progressively heavier solution.

3. Prepare light and heavy acrylamide gel solutions (Table 6.1.10). Use ~12 ml of each solution for five 0.75-mm-thick minigels.

   Adjust volumes if a different thickness or number of gels is needed. Do not add ammonium persulfate until just before use. Deaeration is not recommended for gradient gels.

4. With the outlet and interconnecting valve closed, add the heavy solution to the reservoir chamber. Briefly open the interconnecting valve to let a small amount of heavy solution through to the mixing chamber, clearing the valve of air.

5. Fill the mixing chamber with light solution. Add 4 µl TEMED per 12 ml acrylamide solution to each chamber and mix with a disposable pipet.

Form the gradient and cast the gels

6. Turn on the magnetic stirrer. Open the interconnecting valve and allow the chambers to equilibrate. Then slowly open the outlet port to allow the solution to flow from the gradient maker to the multiple caster by gravity (a peristaltic pump may be used for better control). Adjust the flow rate to 3 to 4 ml/min.

   Faster flow rates are possible and will also produce good gradients. However, a fast flow increases the potential for introduction of bubbles into the caster.

7. Close the outlet port as the last of the gradient solution leaves the mixing chamber, just before air enters the outlet tube. Fill the two chambers with plug solution and slowly open the outlet once again.

8. Allow the plug solution to push the acrylamide in the caster up into the plates. Close the outlet when the plug solution reaches the bottom of the plates.

   A discontinuity between the bottom of the gels and the plug solution will be obvious.

9. Quickly add 100 µl H_2O-saturated isobutyl alcohol to each gel sandwich. Let the gels polymerize undisturbed for ~1 hr.

10. Prepare and pour the stacking gel (see Basic Protocol 2, steps 9 and 10).

Disassemble the system

11. Disconnect the gradient maker, place the caster in a sink, and remove the front faceplate. The plug solution will drain out from the bottom of the caster.

12. Remove the gels (see Basic Protocol 2, step 11).

    Gradient minigels can be stored as described for single-concentration minigels (see Basic Protocol 2, step 11 annotation). For instructions on preparing, loading, and running the gels, see Basic Protocol 2, steps 12 to 17.

SUPPORT PROTOCOL 4

CALCULATING MOLECULAR MASS

Determining the molecular mass of an unknown protein or nucleic acid fragment is straightforward given the use of calibration size standards in the same gel. Typically, a tracking dye such as bromphenol blue is added to the sample prior to loading on the gel (e.g., see recipe for SDS sample buffer). The tracking dye moves ahead of the proteins and serves as a relative mobility marker. A set of protein standards is separated in the same gel as the protein sample containing the unknown. The standards are used to create a standard curve of relative mobility versus size or molecular mass. Although digital image analysis has greatly simplified calculating the mass of an unknown protein separated by electrophoresis, manual assessments of molecular mass are useful and use the same basic calculations.

1. Calculate the relative mobility (R_f) using following formula:

   R_f = distance migrated by protein/distance migrated by marker.

   Placing a molecular mass/R_f mobility acetate overlay calculator (Fig. 6.1.5) on the gel is a quick way to determine R_f. Simply align the top and bottom of the overlay with the top of the gel and the dye front, respectively, to get a read out of R_f.

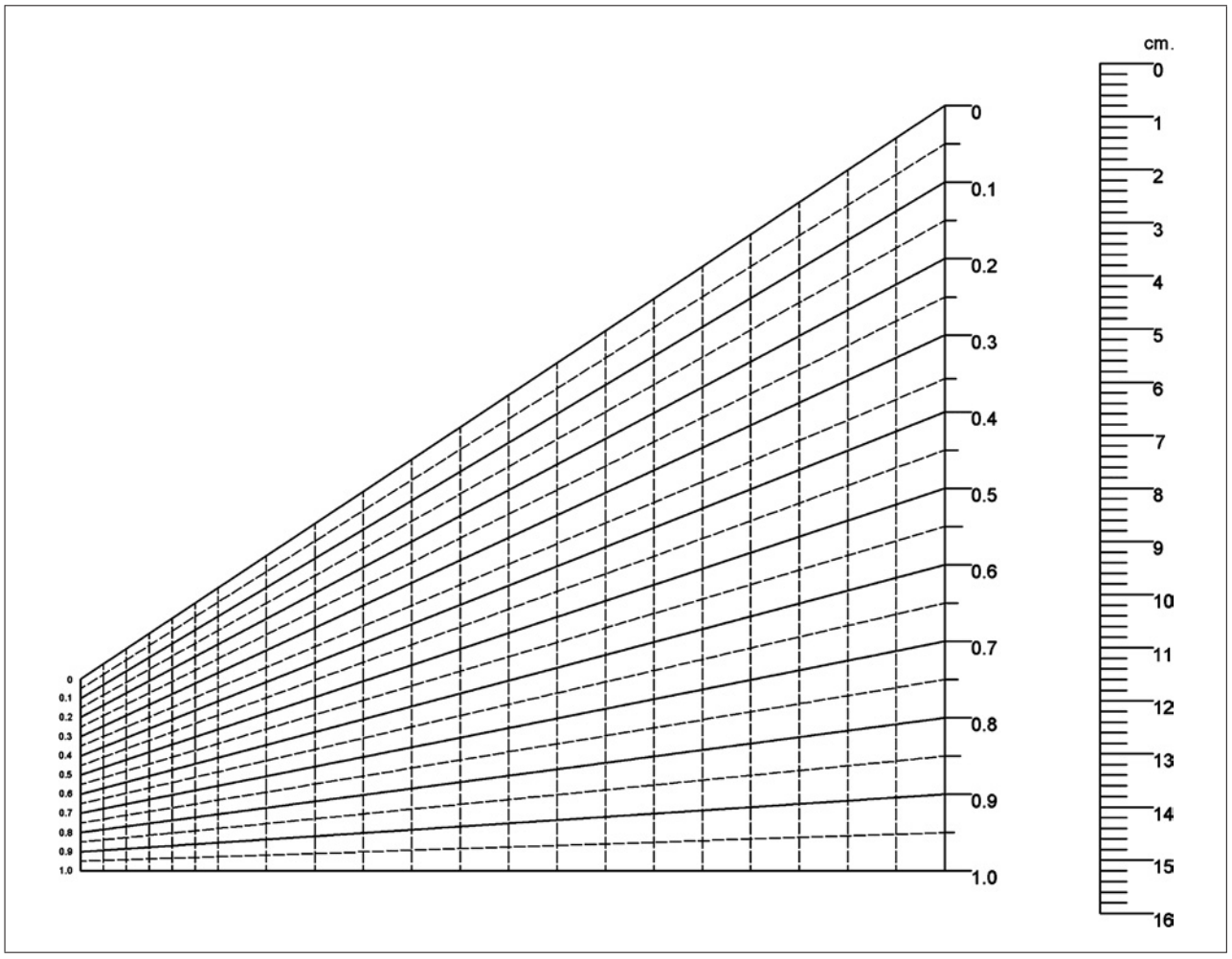

Figure 6.1.5 Example of an R_f calculator. This sheet is copied to transparency film using a paper copier and used as an overlay on the gel. When the transparency is placed on top of the gel, so that the top of the gel aligns with the top of the calculator and the dye front aligns with the bottom of the calculator, the R_f can be read directly off the overlay. Note that the calculator accommodates a range of gel lengths. The overlay should be copied at a 1:1 ratio so that the centimeter scale remains accurate. However, as long as the overlay can fit the top and bottom of the gel, the R_f numbers will be accurate.

2. Plot log protein mass on the *y* axis versus relative mobility of the standards on the *x* axis (Fig. 6.1.6).
3. Perform linear regression using a calculator or analysis program.
4. Use the linear regression equation ($y = mx + b$) to estimate the mass of the unknown:

   Log molecular weight = (slope)(mobility of unknown) + *y* intercept.

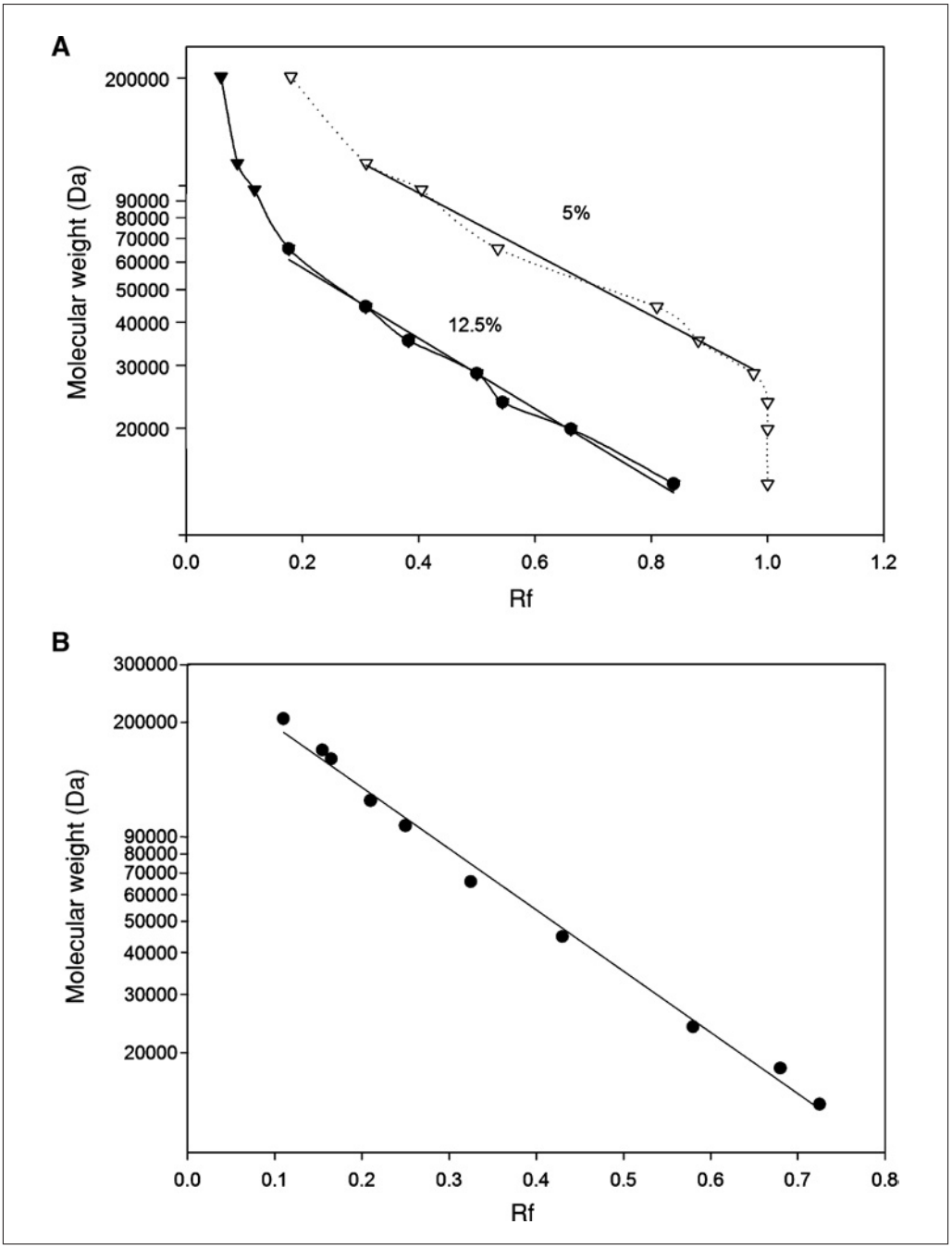

Figure 6.1.6 Standard protein molecular weight curves for **(A)** single concentration (5% and 12.5%) and **(B)** gradient (5% to 20%) gels. Protein standards are separated via SDS-PAGE, visualized by staining with Coomassie blue (*UNIT 6.6*), and measured relative to the dye front to give the relative mobility (R_f). Note the single-concentration gel has a more limited range of linearity than the gradient gel. The standard curve permits the calculation of the molecular weight of an unknown by using the R_f of the unknown to predict the molecular weight.

REAGENTS AND SOLUTIONS

Use Milli-Q water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Anode buffer

121.1 g Tris base (0.2 M final)
500 ml H_2O
Adjust to pH 8.9 with concentrated HCl
Dilute to 5 liters with H_2O
Store at 4°C up to 1 month

Final concentration is 0.2 M Tris·Cl, pH 8.9.

Cathode buffer

12.11 g Tris base (0.1 M final)
17.92 g tricine (0.1 M final)
1 g SDS [0.1% w/v final; recrystallization (see recipe) optional]
Dilute to 1 liter with H_2O
Do not adjust pH
Store at 4°C up to 1 month

Coomassie blue G-250 staining solution

200 ml acetic acid (20% v/v final)
1800 ml H_2O
0.5 g Coomassie blue G-250 (0.025% w/v final)
Mix 1 hr and filter (Whatman no. 1 paper)
Store at room temperature indefinitely

Phosphate/SDS electrophoresis buffer

Dilute 500 ml of 4× phosphate/SDS, pH 7.2 (Table 6.1.8) with H_2O to 2 liters. Store at 4°C up to 1 month.

Final concentrations are 0.1 M sodium phosphate (pH 7.2)/0.1% (w/v) SDS.

Phosphate/SDS sample buffer, 2× (for continuous systems)

0.5 ml 4× phosphate/SDS, pH 7.2 (Table 6.1.8; 20 mM sodium phosphate final)
0.2 g SDS [2% w/v final; recrystallization (see recipe) optional]
0.1 mg bromphenol blue (0.001% w/v final)
0.31 g DTT (0.2 M final)
2.0 ml glycerol (20% v/v final)
Add H_2O to 10 ml and mix

Plug solution

0.125 M Tris·Cl, pH 8.8 (*APPENDIX 2A*)
50% (w/v) sucrose
0.001% (w/v) bromphenol blue
Store at 4°C up to 1 month

Recrystallized SDS (optional)

High-purity SDS is available from several suppliers, but for some sensitive applications (e.g., protein sequencing) recrystallization is useful. Commercially available electrophoresis-grade SDS is usually of sufficient purity for most applications.

continued

Add 100 g SDS to 450 ml ethanol and heat to 55°C. While stirring, gradually add 50 to 75 ml hot H_2O until all SDS dissolves. Add 10 g activated charcoal (Norit 1, Sigma) to solution. After 10 min, filter solution through Whatman no. 5 paper on a Buchner funnel to remove charcoal. Chill filtrate 24 hr at 4°C and 24 hr at −20°C. Collect crystalline SDS on a coarse-frit (porosity A) sintered-glass funnel and wash with 800 ml −20°C ethanol (reagent grade). Repeat crystallization without adding activated charcoal. Dry recrystallized SDS under vacuum overnight at room temperature. Store in a desiccator over phosphorous pentoxide (P_2O_5) in a dark bottle.

If proteins will be electroeluted or electroblotted for protein sequence analysis, it may be desirable to crystallize the SDS twice from ethanol/H_2O (Hunkapiller et al., 1983).

SDS electrophoresis buffer, 5×

15.1 g Tris base (0.125 M final)
72.0 g glycine (0.96 M final)
5.0 g SDS [0.5% w/v final; recrystallization (see recipe) optional]
H_2O to 1000 ml
Dilute to 1× or 2× for working solution, as appropriate

Do not adjust the pH of the stock solution, as the solution is pH 8.3 when diluted. Store at 0° to 4°C until use (up to 1 month).

SDS sample buffer, 2× (for discontinuous systems)

25 ml 4× Tris·Cl/SDS, pH 6.8 (Table 6.1.1)
20 ml glycerol (20% final)
4 g SDS [4% w/v final; recrystallization (see recipe) optional]
2 ml 2-ME *or* 3.1 g DTT (0.2% v/v 2-ME or 0.2 M DTT final)
1 mg bromphenol blue (0.001% w/v final)
Add H_2O to 100 ml and mix
Store in 1-ml aliquots at −70°C

To avoid reducing proteins to subunits (if desired), omit 2-ME or DTT (reducing agent) and add 10 mM iodoacetamide to prevent disulfide interchange.

SDS sample buffer, 6× (for discontinuous systems)

7 ml 4× Tris·Cl/SDS, pH 6.8 (Table 6.1.1)
3.0 ml glycerol (30% v/v final)
1 g SDS [10% w/v final; recrystallization (see recipe) optional]
0.93 g DTT (0.6 M final)
1.2 mg bromphenol blue (0.012% w/v final)
Add H_2O to 10 ml (if needed)
Store in 0.5-ml aliquots at −70°C

Tricine sample buffer, 2×

2 ml 4× Tris·Cl/SDS, pH 6.8 (Table 6.1.1; 0.1 M)
2.4 ml (3.0 g) glycerol (24% v/v final)
0.8 g SDS [8% w/v final; recrystallization (see recipe) optional]
0.31 g DTT (0.2 M final)
2 mg Coomassie blue G-250 (0.02% w/v final)
Add H_2O to 10 ml and mix

COMMENTARY

Background Information

Although electrophoresis has been studied for over two centuries, Arne Tiselius (University of Uppsala, Sweden) put the technique on the map with his Ph.D. thesis in 1930, which demonstrated moving zones of serum proteins in a special square-shaped, free-solution electrophoresis cell. His original work showed for the first time that protein components in serum could be separated, giving the now familiar designations for α, β, and γ globulin. Arne Tiselius was awarded the Nobel Prize in Chemistry in 1948 (*http://nobelprize.org/chemistry/laureates/1948/index.html*) "... for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins."

Electrophoresis is the movement of a charged particle, including large molecules such as DNA and proteins, in a liquid medium under the influence of an electric field. The driving force (QE) on the charged molecule is a product of the charge (Q) and the electric field (E) across the separation gel. Larger proteins move more slowly, as do proteins with a lower net charge. Furthermore, if a protein is in native (compact) form it will migrate more quickly than the same protein fully denatured and extended, where it experiences more frictional resistance with the surrounding medium. Resistance in electrophoresis is defined as $f = 6\pi rv\eta$, where f is the resistance of the medium to electrophoretic movement, r the radius of the protein (assumed to be a sphere), v the electrophoretic velocity, and η the viscosity of the fluid. The elements contributing to driving force and resistance together indicate that protein charge, size, shape, solution viscosity, and applied voltage are key factors influencing electrophoretic separation.

Electrophoresis is used to separate complex mixtures of proteins (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates), investigate subunit compositions, and verify homogeneity of protein samples (Table 6.1.12). It can also serve to purify proteins for use in further applications. In polyacrylamide gel electrophoresis (PAGE), proteins migrate in response to an electrical field through pores in a gel matrix consisting of polymers of cross-linked acrylamide. The pore size is determined by acrylamide concentration. The combination of gel pore size and protein charge, size, and shape determines the migration rate of the protein.

Polyacrylamide gels form after polymerization of monomeric acrylamide into polymeric polyacrylamide chains and cross-linking of the chains by *N,N′*-methylenebisacrylamide (Fig. 6.1.7). The polymerization reaction is initiated by the addition of ammonium persulfate, and the reaction is accelerated by TEMED, which catalyzes the formation of free radicals from ammonium persulfate. Because oxygen inhibits the polymerization process, deaerating the gel solution before the polymerization catalysts are added will speed up polymerization; deaeration is not recommended for the gradient gel protocols because slower polymerization facilitates casting of gradient gels.

Precast gels for commonly used vertical minigel and standard-sized SDS-PAGE apparatuses are available from several manufacturers (Table 6.1.4). Flatbed (horizontal) isoelectric focusing (IEF) and SDS-PAGE gels are not listed. Hoefer supplies a range of horizontal gels for a variety of applications and should be consulted for further information. When using precast gels, pay strict attention to shelf life. In general, manufacturers overrate the shelf life, and the sooner the gels are used, the better. When reasonably fresh, precast gels provide excellent resolution that is as good or better than a typical gel cast in the laboratory.

The most widely used method for discontinuous gel electrophoresis is the system described by Laemmli (1970). This is the denaturing (SDS) discontinuous method used in Basic Protocol 1. A discontinuous buffer system uses buffers of different pH and composition to generate a discontinuous pH and voltage gradient in the gel. Because the discontinuous gel system concentrates the proteins in each sample into narrow bands, the applied sample may be more dilute than that used for continuous electrophoresis.

In the discontinuous system the sample first passes through a stacking gel, which has large pores. The stacking gel buffer contains chloride ions (called the leading ions) whose electrophoretic mobility is greater than the mobility of the proteins in the sample. The electrophoresis buffer contains glycine ions (called the trailing ions) whose electrophoretic mobility is less than the mobility of the proteins in the sample. The net result is that the faster migrating ions leave a zone of lower conductivity between themselves and the migrating protein. The higher voltage gradient in this zone allows the proteins to move faster and to "stack" in the zone between the leading

Table 6.1.12 Key Applications of Protein Electrophoresis[a]

Technique	Separation principle	Application	For more information
Native PAGE	Native charge, size, and shape	Purification, determination of native protein size, and identification of protein complexes	*UNIT 6.5*
1-D SDS-PAGE	Size dependent: SDS imparts a negative charge to proteins, giving a constant charge to mass ratio	Size estimation, purity check, purification, subunit composition, protein expression	*UNIT 6.1*
IEF	Intrinsic charge with both native and denatured proteins	Purification, purity check, isoenzyme analysis	Ploegh (1995)
2-D SDS-PAGE	Isoelectric point in the first dimension, size in the second	Protein expression, purification, posttranslational analysis, proteomics	*UNIT 6.4*

[a]Abbreviations: 1-D, one-dimensional; 2-D, two-dimensional; IEF, isoelectric focusing.

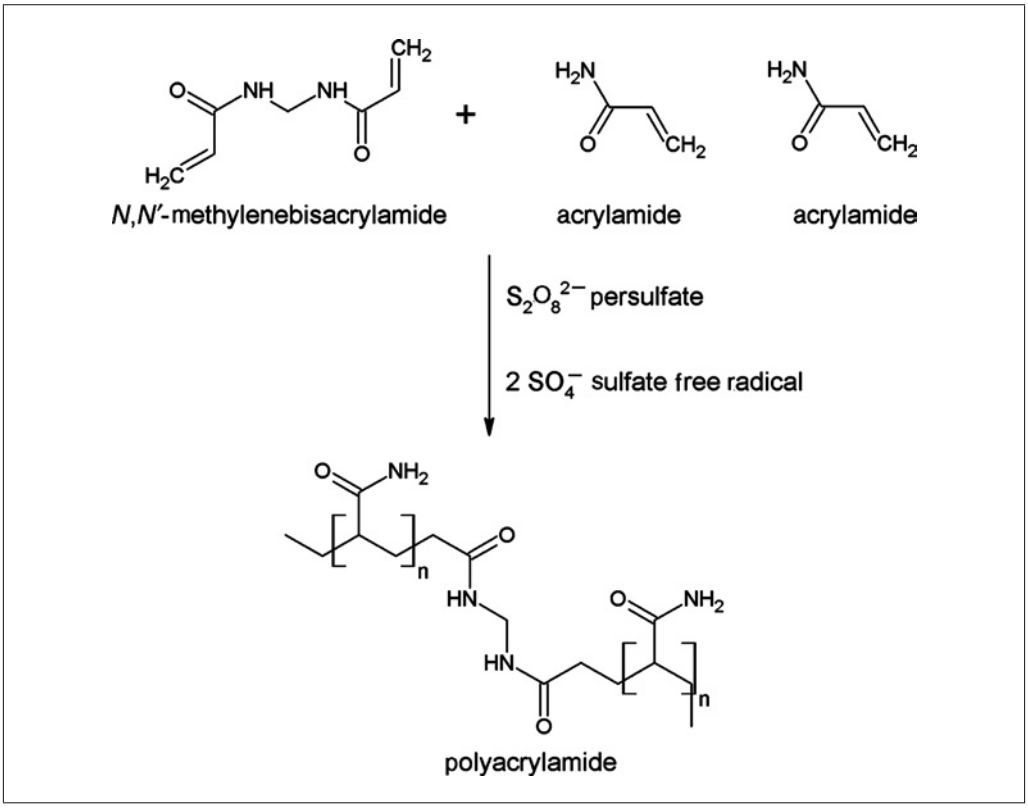

Figure 6.1.7 Structures of acrylamide and bisacrylamide and the associated reaction producing the polyacrylamide matrix used for protein separation.

and trailing ions. After leaving the stacking gel, the protein enters the separating gel. The separating gel has a smaller pore size, a higher salt concentration, and higher pH compared to the stacking gel. In the separating gel, the glycine ions migrate past the proteins, and the proteins are separated according to either molecular size in a denaturing gel (containing SDS) or molecular shape, size, and charge in a nondenaturing gel.

Proteins are denatured by heating in the presence of a low-molecular-weight thiol

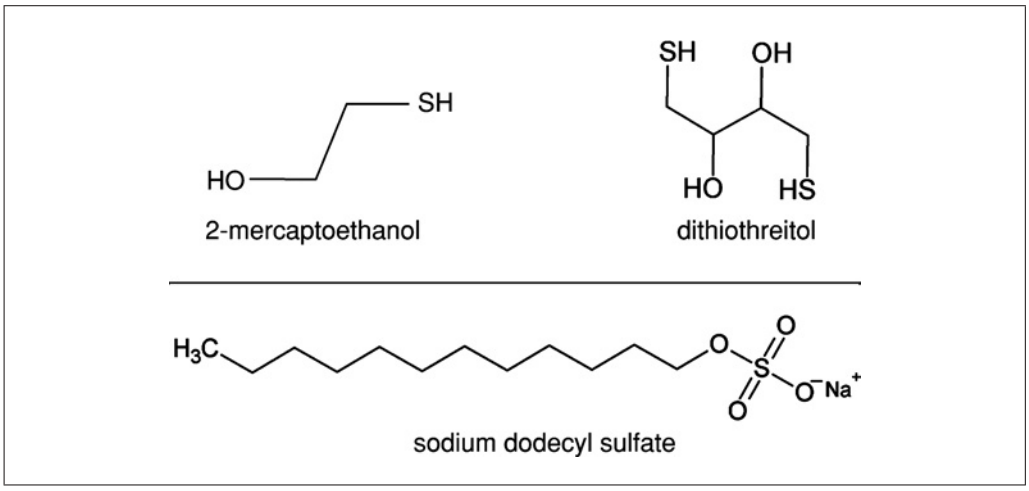

Figure 6.1.8 Structures of sodium dodecyl sulfate, dithiothreitol, and 2-mercaptoethanol: used to break disulfide bonds in proteins so they are fully denatured.

(2-ME or DTT) and SDS (Fig. 6.1.8). Most proteins bind SDS in a constant-weight ratio, leading to identical charge densities for the denatured proteins. Thus, the SDS-protein complexes migrate in the polyacrylamide gel according to size, not charge. Most proteins are resolved on polyacrylamide gels containing from 5% to 15% acrylamide and 0.2% to 0.5% bisacrylamide (see Table 6.1.1). The relationship between the relative mobility and log molecular weight is linear over these ranges (Fig. 6.1.6). With the use of plots like those shown in Figure 6.1.6, the molecular weight of an unknown protein (or its subunits) may be determined by comparison with known protein standards (Table 6.1.2). In general, all of the procedures in this unit are suitable for radiolabeled and biotinylated proteins without modification.

Basic Protocol 1 relies on denaturing proteins in the presence of SDS and 2-ME or DTT. Under these conditions, the subunits of proteins are dissociated and their biological activities are lost. A true estimate of a protein's molecular size can be made by comparing the relative mobility of the unknown protein to proteins in a calibration mixture (Support Protocol 4). Gradient gels (Alternate Protocol 5) simplify molecular-weight determinations by producing a linear relationship between log molecular weight of the protein and log % T over a much wider size range than single-concentration gels. Although percent acrylamide monomer is a more common measure of gel concentration, % T, the percentage of total monomer (acrylamide plus bisacrylamide) in the solution or gel, is used for molecular weight calculations in gradient gels. The % T of a stained protein is estimated assuming the acrylamide gradient is linear. For example, proteins in the gel shown in Figure 6.1.9 were separated in a 5.1% to 20.5% T acrylamide gradient. The % T of the point halfway through the resolving gel is 12.5% T. Simply plotting log molecular mass versus distance moved into the gel (or R_f) also produces a relatively linear standard curve over a fairly wide size range.

If two proteins have identical molecular sizes, they more than likely will not be resolved with one-dimensional SDS-PAGE, and two-dimensional SDS-PAGE should be considered. Unusual protein compositions can cause anomalous mobilities during electrophoresis (see Critical Parameters and Troubleshooting), but similar-sized proteins of widely different amino acid composition or structure may still be resolved from one another using one-dimensional SDS-PAGE. Purified protein complexes or multimeric proteins consisting of subunits of different molecular size will be resolved into constituent polypeptides. Comparison of the protein bands obtained under nonreducing and reducing conditions provides information about the molecular size of disulfide cross-linked component subunits. The individual polypeptides can be isolated by electroelution or electroblotting, and the amino acid sequences can be determined.

Both the tricine (Schagger and von Jagow, 1987) and the modified Laemmli (Okajima et al., 1993) peptide separation procedures presented here (Alternate Protocols 1 and 2) are simple to set up and provide resolution down to 5 kDa. To separate peptides below 5 kDa, the tricine procedure must be modified by preparing a 16.5% T, 2.7% C resolving gel that uses a 10% T spacer gel between the stacking

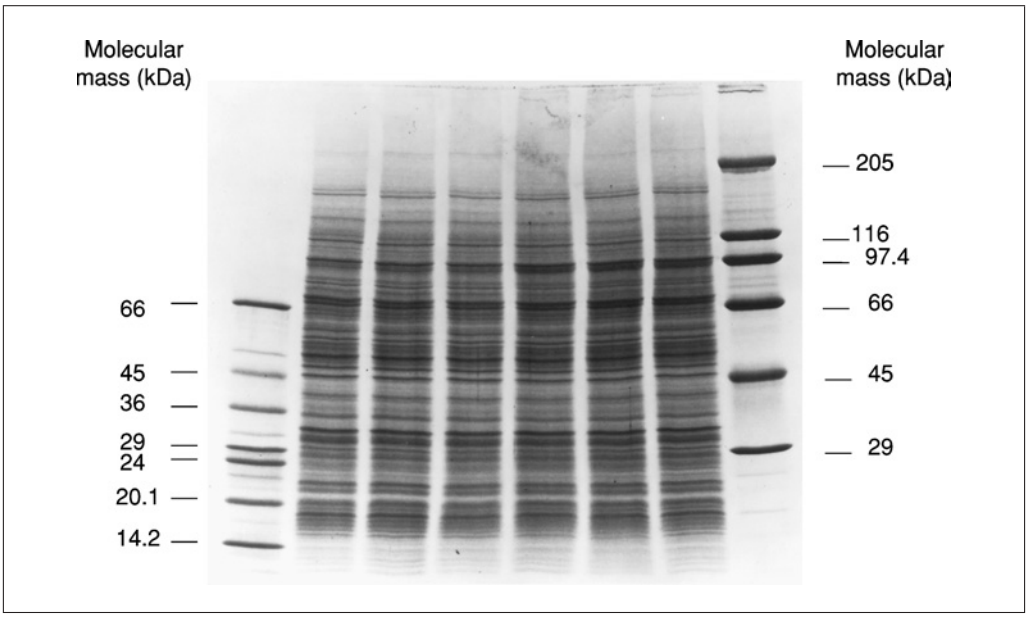

Figure 6.1.9 Separation of membrane proteins by 5.1% to 20.5% T polyacrylamide gradient SDS-PAGE. Approximately 30 μl of 1× SDS sample buffer containing 30 μg of Alaskan pea (*Pisum sativum*) membrane proteins was loaded in wells of a 14 × 14–cm, 0.75-mm-thick gel. Standard proteins were included in the outside lanes. The gel was run at 4 mA for ≈15 hr.

and resolving gel (Schagger and von Jagow, 1987). The % C is the percentage of cross-linker (bisacrylamide) in the total monomer (acrylamide plus bisacrylamide).

Continuous electrophoresis, where the same buffer is used throughout the tank and gel, is popular because of its versatility and simplicity. The phosphate system described in Alternate Protocol 3 is based on that of Weber et al. (1972). Although unable to produce the high-resolution separations of the discontinuous SDS-PAGE procedures, continuous SDS-PAGE uses fewer solutions with one basic buffer and no stacking gel. Artifacts are also less likely to occur in continuous systems. Pepsin, for example, migrates anomalously on Laemmli-based discontinuous SDS-PAGE but has the expected mobility after electrophoresis in the phosphate-based continuous system described here. This is also true of cross-linked proteins.

Multiple gel casting (Support Protocols 1 to 3) is appropriate when gel-to-gel consistency is paramount or when the number of gels processed exceeds five a week. The variety of multiple gel casters, gradient makers, and inexpensive pumps available from major suppliers simplifies the process of casting gels in the laboratory. Alternatively, precast gradient gels are available for most major brands of gel apparatuses (Table 6.1.4).

Minigels (Basic Protocol 2) are generally considered to be in the 8 × 10–cm size range, although there is considerable variation in exact size. Every technique that is used on larger systems can be translated with little difficulty into the minigel format. This includes standard and gradient SDS-PAGE and separations for immunoblotting and peptide sequencing. Two-dimensional SDS-PAGE electrophoresis also adapts well, but here the limitation of separation area becomes apparent; for high-resolution separations, large-format gels are required. Gradient minigels (Support Protocol 3) are popular due to the combination of separation range and resolution (Matsudaira and Burgess, 1978). They are particularly useful for separation of proteins prior to peptide sequencing.

Mylar support (GelBond) provides a practical way of casting, running, and, staining extremely thin gels. When gels <0.75 mm thick are used, reagents have much better access both into and out of the gel, reducing staining time in both Coomassie blue and silver staining. Double and broadened images caused by differential migration of the protein across the thickness of the gel are minimized, improving resolution.

Critical Parameters and Troubleshooting

If an electrophoretically separated protein will be electroeluted or electroblotted for sequence analysis, the highest-purity reagents available should be used. If necessary, SDS

can be purified by recrystallization following the procedure given in Reagents and Solutions.

If the gels polymerize too fast, the amount of ammonium persulfate should be reduced by one-third to one-half. If the gels polymerize too slowly or fail to polymerize all the way to the top, use fresh ammonium persulfate or increase the amount of ammonium persulfate by one-third to one-half. The overlay should be added slowly down the spacer edge to prevent the overlay solution from crashing down and disturbing the gel interface.

After a separating gel is poured, it may be stored with an overlay of the same buffer used in the gel. Immediately prior to use, the stacking gel should be poured; otherwise, there will be a gradual diffusion-driven mixing of buffers between the two gels, which will cause a loss of resolution.

The protein of interest should be present in 0.2- to 1-μg amounts in a complex mixture of proteins if the gel will be stained by Coomassie blue (*UNIT 6.6*). Typically, 30 to 50 μg of a complex protein mixture in a total volume of <20 μl is loaded on a 0.75-mm-thick slab gel (16 cm, 10 wells).

When casting multiple gradient gels, eliminate all bubbles in the outlet tubing of the gradient maker. If air bubbles get into the outlet tube, they may flow into the caster and then up through the gradient being poured, causing an area of distortion in the polymerized gel. Air bubbles are not so great a problem when casting single gradient gels from the top. As the gels are cast, the stirrer must be slowed so that the vortex in the mixing chamber does not allow air to enter the outlet.

Uneven heating of the gel causes differential migration of proteins, with the outer lanes moving more slowly than the center lanes (called smiling). Increased heat transfer eliminates smiling and can be achieved by filling the lower buffer chamber with buffer all the way to the level of the sample wells, by maintaining a constant temperature between 10° to 20°C, and by stirring the lower buffer with a magnetic stirrer. Alternatively, decrease the heat load by running at a lower current.

If the tracking dye band is diffuse, prepare fresh buffer and acrylamide monomer stocks. If the protein bands are diffuse, increase the current by 25% to 50% to complete the run more quickly and minimize band diffusion, use a higher percentage of acrylamide, or try a gradient gel. Lengthy separations using gradient gels generally produce good results (Fig. 6.1.9). Check for possible proteolytic degradation that may cause loss of high-molecular-weight bands and create a smeared banding pattern.

If there is vertical streaking of protein bands, decrease the amount of sample loaded on the gel, further purify the protein of interest to reduce the amount of contaminating protein applied to the gel, or reduce the current by 25%. Another cause of vertical streaking of protein bands is precipitation, which can sometimes be eliminated by centrifuging the sample or by reducing the percentage of acrylamide in the gel.

Proteins can migrate faster or slower than their actual molecular weight would indicate. Abnormal migration is usually associated with a high proportion of basic or charged amino acids (Takano et al., 1988). Other problems can occur during isolation and preparation of the protein sample for electrophoresis. Proteolysis of proteins during cell fractionation by endogenous proteases can cause subtle band splitting and smearing in the resulting electrophoretogram (electrophoresis pattern). Many endogenous proteases are very active in SDS sample buffers and will rapidly degrade the sample; thus, first heating the samples to 70° to 100°C for 3 min is recommended.

In some cases, heating to 100°C in sample buffer will cause selective aggregation of proteins, creating a smeared layer of Coomassie blue–stained material at the top of the gel (Gallagher and Leonard, 1987). To avoid heating artifacts and also prevent proteolysis, the use of specific protease inhibitors during protein isolation and/or lower heating temperatures (70° to 80°C) have been effective (Dhugga et al., 1988).

Although continuous gels suffer from poor band sharpness, they are less prone to artifacts caused by aggregation and protein cross-linking. If streaking or aggregation appear to be a problem with the Laemmli system, then the same sample should be subjected to continuous SDS-PAGE to see if the problem is intrinsic to the Laemmli gel or the sample.

If the protein bands spread laterally from gel lanes, the time between applying the sample and running the gel should be reduced in order to decrease the diffusion of sample out of the wells. Alternatively, the acrylamide percentage should be increased in the stacking gels from 4% to 4.5% or 5% acrylamide, or the operating current should be increased by 25% to decrease diffusion in the stacking gel. Use caution when adding 1× SDS electrophoresis buffer to the upper buffer chamber. Samples

can get swept into adjacent wells and onto the top of the well arm.

If the protein bands are uneven, the stacking gel may not have been adequately polymerized. This can be corrected by deaerating the stacking gel solution thoroughly or by increasing the ammonium persulfate and TEMED concentrations by one-third to one-half. Another cause of distorted bands is salt in the protein sample, which can be removed by dialysis, gel filtration, or precipitation. Skewed protein bands can be caused by an uneven interface between the stacking and separating gels, which can be corrected by starting over and being careful not to disturb the separating gel while overlaying with isobutyl alcohol.

If a run takes too long, the buffers may be too concentrated or the operating current too low. If the run is too short, the buffers may be too dilute or the operating current too high.

If double bands are observed, the protein may be partially oxidized or partially degraded. Oxidation can be minimized by increasing the 2-ME concentration in the sample buffer or by preparing a fresh protein sample. If fewer bands than expected are observed and there is a heavy protein band at the dye front, increase the acrylamide percentage in the gel.

Anticipated Results

Polyacrylamide gel electrophoresis done under denaturing and reducing conditions should resolve any two proteins, except two of identical size. Resolution of proteins in the presence of SDS is a function of gel concentration and the size of the proteins being separated. Under nondenaturing conditions, the biological activity of a protein will be maintained.

Comparison of reducing and nonreducing denaturing gels can also provide valuable information about the number of disulfide cross-linked subunits in a protein complex. If the subunits are held together by disulfide linkages, the protein will separate in denaturing gels as a complex or as smaller-sized subunits under nonreducing or reducing conditions, respectively. However, proteins separated on nonreducing denaturing gels appear more diffuse and exhibit less overall resolution than those separated on reducing gels.

Gradient gels provide superior protein-band sharpness and resolve a larger size range of proteins, making them ideal for most types of experiments in spite of being more difficult to prepare. Molecular-weight calculations are simplified because of the extended linear relationship between size and protein position in the gel. Increased band sharpness of both high- and low-molecular-weight proteins on the same gel greatly simplifies survey experiments, such as gene expression studies where the characteristics of the responsive protein are not known. Furthermore, the increased resolution dramatically improves autoradiographic analysis. Preparation of gradient gels is straightforward, although practice with gradient solutions containing dye is recommended. The gradient gels can be stored for several days at 0° to 4°C before casting the stacking gel.

Time Considerations

Preparation of separating and stacking gels requires 2 to 3 hr. Gradient gels generally take 5 min to cast singly. Casting multiple single-concentration gels requires an additional 10 min for assembly. Casting multiple gradient gels takes 15 to 20 min plus assembly time. It takes 4 to 5 hr to run a 14 × 14–cm, 0.75-mm gel at 15 mA (70 to 150 V), and 3 to 4 hr to run a 0.75-mm gel at 20 mA (80 to 200 V). Overnight separations of ~12 hr require 4 mA per 0.75-mm gel. It takes 4 to 5 hr to run a 1.5-mm gel at 30 mA. Electrophoresis is normally performed at 15° to 20°C, with the temperature held constant using a circulating water bath. For air-cooled electrophoresis units, lower currents and thus longer run times are recommended.

It takes ~1 hr to run a 0.75-mm minigel at 20 mA (100 to 120 V). Separation times are not significantly different for gradient minigels.

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An excellent book describing gel electrophoresis of proteins.

Immunoblotting and Immunodetection

Immunoblotting (often referred to as western blotting) is used to identify specific antigens recognized by polyclonal or monoclonal antibodies. Protein samples are solubilized, usually with sodium dodecyl sulfate (SDS) and reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME). Following solubilization, the material is separated by SDS-PAGE (*UNIT 6.1*). The antigens are then electrophoretically transferred in a tank (see Basic Protocol 1) or a semidry transfer apparatus (see Alternate Protocol 1) to a nitrocellulose, PVDF, or nylon membrane, a process that can be monitored by reversible staining (see Support Protocol 1) or by Ponceau S staining (see Support Protocol 2). Previously stained gels may also be blotted (see Alternate Protocol 2).

The transferred proteins are bound to the surface of the membrane, providing access for reaction with immunodetection reagents. All remaining binding sites are blocked by immersing the membrane in a solution containing either a protein or detergent blocking agent. After probing with the primary antibody, the membrane is washed and the antibody-antigen complexes are identified with horseradish peroxidase (HRPO) or alkaline phosphatase enzymes coupled to the secondary anti-IgG antibody (e.g., goat anti–rabbit IgG). The enzymes are attached directly (see Basic Protocol 2) or via an avidin-biotin bridge (see Alternate Protocol 3) to the secondary antibody. Chromogenic or luminescent substrates (see Basic Protocol 3 and Alternate Protocol 4) are then used to visualize the activity. Finally, membranes may be stripped and reprobed (see Support Protocol 3).

NOTE: When handling gels and membranes wear powder-free gloves.

PROTEIN BLOTTING WITH TANK TRANSFER SYSTEMS

BASIC PROTOCOL 1

In this procedure, blotting is performed in a tank of buffer with the gel in a vertical orientation, completely submerged between two large electrode panels. In some systems up to four gels can be transferred at one time. For difficult-to-transfer proteins (>100 kDa or hydrophobic; e.g., myosin), tank blotting is preferable to semidry systems (see Basic Protocol 2) because prolonged transfers are possible without buffer depletion. However, transfers >1 hr at high power require cooling using a heat exchanger and a circulating water bath that can maintain a constant transfer temperature of 10° to 20°C.

Materials

Samples for analysis
Protein molecular weight standards (*UNIT 6.1*), prestained (Sigma or Bio-Rad) or biotinylated (Vector Labs or Sigma)
Transfer buffer (see recipe)

Powder-free gloves
Scotch-Brite pads (3M) or equivalent sponge
Whatman 3MM filter paper or equivalent
Transfer membrane: 0.45-µm nitrocellulose (Millipore or Schleicher & Schuell), PVDF (Millipore Immobilon P), neutral nylon (Pall Biodyne A), *or* positively charged nylon (Pall Biodyne B; Bio-Rad Zetabind) membrane
Electroblotting apparatus (EC Apparatus, Bio-Rad, or Amersham Pharmacia Biotech)
Indelible pen (e.g., Paper-Mate ballpoint) or soft lead pencil

Additional reagents and equipment for gel electrophoresis (*UNIT 6.1*) and staining proteins in gels and on membranes (see Support Protocol 1)

NOTE: Deionized, distilled water should be used throughout this protocol.

Contributed by Sean Gallagher, Scott E. Winston, Steven A. Fuller, and John G.R. Hurrell
Current Protocols in Cell Biology (1998) 6.2.1-6.2.20
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Electrophorese the protein sample

1. Prepare antigenic samples and separate proteins using small or standard-sized one- or two-dimensional gels (*UNIT 6.1*). Include prestained or biotinylated protein molecular weight standards in one or more gel lanes.

   The protein markers will transfer to the membrane and conveniently indicate membrane orientation and sizes of proteins after immunostaining.

   A variety of gel sizes and percentages of acrylamide can be used (UNIT 6.1). Most routinely used are either 14 cm × 14 cm × 0.75–mm gels or 8 cm × 10 cm × 0.75–mm minigels. Acrylamide concentrations vary from 5% to 20%, but are usually in the 10% to 15% range.

Assemble the immunoblot sandwich

2. When electrophoresis is complete, disassemble gel sandwich and remove stacking gel. Equilibrate gel 30 min at room temperature in transfer buffer.

   Oil from hands blocks the transfer.

   Match the appropriate transfer buffer to the membrane (see Reagents and Solutions).

   Gel equilibration is required to prevent a change in the size of the gel during transfer. Any shift in gel dimension will result in a blurred transfer pattern.

3. Assemble transfer sandwich in a tray large enough to hold the plastic transfer cassette. Fill with transfer buffer so that cassette is covered.

   The transfer cassette should be assembled under buffer to minimize trapping of air bubbles. Use Figure 6.2.1 as a guide to assembly.

4. On bottom half of plastic transfer cassette, place Scotch-Brite pad or sponge, followed by a sheet of filter paper cut to same size as gel and prewet with transfer buffer.

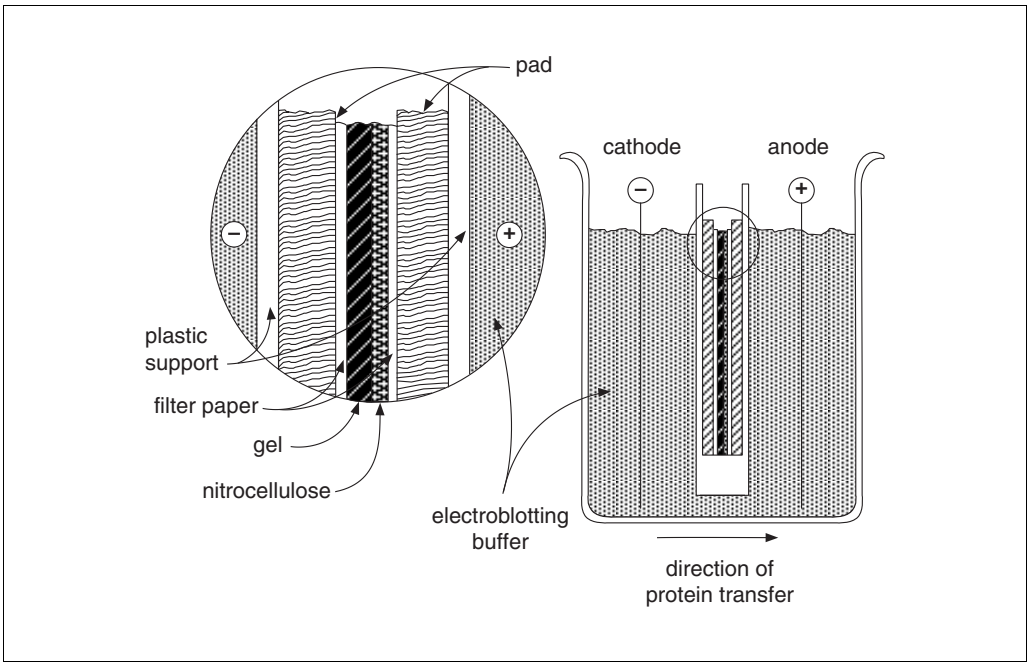

Figure 6.2.1 Immunoblotting with a tank blotting unit. The polyacrylamide gel containing the protein is laid on a sheet of filter paper. The uncovered side of the gel is overlaid with a sheet of membrane precut to the size of the gel plus 1 to 2 mm on each edge, then this membrane is overlaid with another sheet of filter paper. The filter paper containing the gel and membrane is sandwiched between Scotch-Brite pads. This sandwich is placed in a plastic support, and the entire assembly is placed in a tank containing transfer buffer. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Charged proteins are transferred electrophoretically from the gel onto the membrane. Transfer is achieved by applying a voltage of 100 V for 1 to 2 hr (with cooling) or 14 V overnight.

5. Place gel on top of filter paper. The side of the gel touching the paper arbitrarily becomes the cathode side of the gel (i.e., ultimately toward the negative electrode when positioned in the tank). Remove any air bubbles between gel and filter paper by gently rolling a test tube or glass rod over surface of gel.

   Any bubbles between the filter paper, gel, and membrane will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

   Proteins are usually negatively charged in transfer buffer and move toward the positive anode. However, some proteins may be positively charged. An additional membrane placed on the cathode side of the gel will bind these proteins.

6. Prepare transfer membrane. Cut membrane to same size as gel plus 1 to 2 mm on each edge. Place into distilled water slowly, with one edge at a 45° angle. Equilibrate 10 to 15 min in transfer buffer.

   The water will wick up into the membrane, wetting the entire surface. If it is inserted too quickly into the water, air gets trapped and will appear as white blotches in the membrane. Protein will not transfer onto these areas.

   This wetting procedure works for nitrocellulose and nylon membranes only. PVDF membranes are hydrophobic and will not wet simply from being placed into distilled water or transfer buffer. For these membranes, first immerse 1 to 2 sec in 100% methanol, then equilibrate 10 to 15 min with transfer buffer. Do not let membrane dry out at any time. If this occurs, wet once again with methanol and transfer buffer as described above.

7. Moisten surface of gel with transfer buffer. Place prewetted membrane directly on top side of gel (i.e., anode side) and remove all air bubbles as in step 5.

   Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

   The use of 0.2-μm membranes may improve retention of smaller-molecular-weight proteins.

8. Wet another piece of Whatman 3MM filter paper, place on anode side of membrane, and remove all air bubbles. Place another Scotch-Brite pad or sponge on top of this filter paper.

9. Complete assembly by locking top half of the transfer cassette into place (Fig. 6.2.1).

   It is important to orient the sandwich so that the membrane faces the anode (positively charged) side of the tank.

Transfer proteins from gel to membrane

10. Fill tank with transfer buffer and place transfer cassette containing sandwich into electroblotting apparatus in correct orientation. Connect leads of power supply to corresponding anode and cathode sides of electroblotting apparatus.

    Transfer buffer should cover the electrode panels but should not touch the base of the banana plug.

11. Electrophoretically transfer proteins from gel to membrane for 30 min to 1 hr at 100 V with cooling or overnight at 14 V (constant voltage), in a cold room.

    Transfer time is dependent on the thickness and the percent acrylamide of the gel, as well as the size of the protein being transferred. In general, proteins are transferred within 1 to 6 hr, but high-molecular-weight molecules may take longer. Overnight transfer at low voltage is reliable and convenient. Cooling (at 10° to 20°C) is required for transfers >1 hr at high power. Heat exchanger cooling cores using a circulating water bath are placed into the transfer unit for cooling.

12. Turn off the power supply and disassemble the apparatus. Remove membrane from blotting apparatus and note orientation by cutting a corner or marking with a soft lead pencil or Paper-Mate ballpoint pen.

    Many ballpoint inks come off, but Paper-Mate stays on the membrane.

    Membranes can be dried and stored in resealable plastic bags at 4°C for 1 year or longer at this point. Prior to further processing, dried PVDF membranes must be placed into a small amount of 100% methanol to wet the membrane, then in distilled water to remove the methanol.

13. Stain gel for total protein with Coomassie blue to verify transfer efficiency. If desired, stain membrane reversibly to visualize transferred proteins (see Support Protocol 1), or irreversibly with Coomassie blue, India ink, naphthol blue, or colloidal gold.

    These staining procedures are incompatible with nylon membranes.

    If membrane shows significant staining on the backside, either the gel was heavily overloaded or the membrane has poor protein-binding capacity (see Troubleshooting). In either case, protein-binding sites on the side facing the gel are saturated, allowing protein to migrate to the other side of the membrane. Nitrocellulose in particular will show diminished binding capacity with age or poor storage conditions (e.g., high temperature and humidity). In addition, some proteins simply do not bind well to a particular matrix. By using several membrane sheets in place of one, the protein can be detected as it passes through each consecutive sheet. This will give an indication of how efficiently the membrane binds to a particular protein.

14. Proceed with immunoprobing and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 3 and 4).

ALTERNATE PROTOCOL 1

PROTEIN BLOTTING WITH SEMIDRY SYSTEMS

Even and efficient transfer of most proteins is also possible with semidry blotting, a convenient alternative to tank transfer systems. Instead of being placed vertically into a tank filled with transfer buffer, the gel is held horizontally between buffer-saturated blotting paper that is in contact with the electrodes (Fig. 6.2.2), greatly reducing the amount of buffer required. The electrodes are close together, giving high field strengths and rapid transfer with a standard electrophoresis power supply. Prolonged transfers (>1 hr) are not recommended; tank blotting (see Basic Protocol 1) should be used for proteins that require long blotting times for efficient transfer.

Additional Materials *(also see Basic Protocol 1)*

Six sheets of Whatman 3MM filter paper or equivalent, cut to size of gel and saturated with transfer buffer
Semidry transfer unit (Amersham Pharmacia Biotech, Bio-Rad, or Sartorius)

1. Prepare samples and separate proteins using small or standard-sized one- or two-dimensional gels (*UNIT 6.1*).

   Because transfer efficiency depends on many factors (e.g., gel concentration and thickness, protein size, shape, and net charge) results may vary. Below is a guideline for 0.75–mm–thick SDS-PAGE gels transferred by semidry blotting.

Percent acrylamide (resolving gel)	Size range transferred (~100% efficiency)
5–7	29–150 kDa
8–10	14–66 kDa
13–15	<36 kDa
18–20	<20 kDa

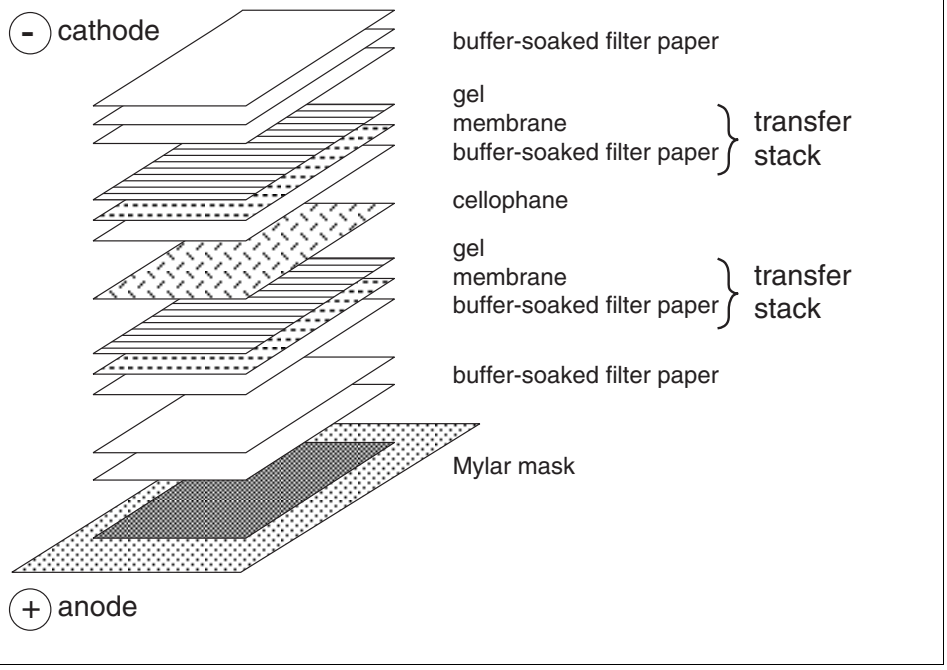

Figure 6.2.2 Immunoblotting with a semidry transfer unit. Generally, the lower electrode is the anode, and one gel is transferred at a time. A Mylar mask (optional in some units) is put in place on the anode. This is followed by three sheets of transfer buffer–soaked filter paper, the membrane, the gel, and finally, three more sheets of buffer-soaked filter paper. To transfer multiple gels, construct transfer stacks as illustrated, and separate each with a sheet of porous cellophane. For transfer of negatively charged protein, the membrane is positioned on the anode side of the gel. For transfer of positively charged protein, the membrane is placed on the cathode side of the gel. Transfer is achieved by applying a maximum current of 0.8 mA/cm^2 of gel area. For a typical minigel (8 × 10 cm) and standard-sized gel (14 × 14 cm), this means 60 and 200 mA, respectively.

2. Prepare transfer membrane (see Basic Protocol 1, step 6).
3. Disassemble gel sandwich. Remove and discard stacking gel.

   Equilibration of the separating gel with transfer buffer is not normally required for semidry blotting, but it may improve transfer in some cases.

4. Place three sheets of filter paper saturated with transfer buffer on the anode (Fig. 6.2.2).

   Most transfer units are designed so that negatively charged proteins move downward toward either a platinum or graphite positive electrode (anode).

   CAPS transfer buffer, pH 10.5 (see recipe for transfer buffer) can be used in place of the Tris/glycine/methanol transfer buffer of Basic Protocol 1. CAPS buffer should be used if the protein is to be sequenced right on the membrane (Moos, 1992), as glycine will interfere with this procedure.

   The filter paper should be cut to the exact size of the gel. This forces the current to flow only through the gel and not through overlapping filter paper. Some manufacturers (e.g., Amersham Pharmacia Biotech) recommend placing a Mylar mask on the lower platinum anode. With an opening that is slightly less than the size of the gel, the mask forces the current to flow through the gel and not the surrounding electrode area during transfer.

5. Place equilibrated transfer membrane on top of filter paper stack. Remove all bubbles between membrane and filter paper by rolling a test tube over surface of membrane.

   Any bubbles in the filter paper stack or between the filter paper, membrane, and gel will block current flow and prevent protein transfer. This problem is indicated on the membrane by sharply defined white areas devoid of transferred protein.

6. Place gel on top of membrane. Gently roll a test tube over surface of gel to ensure intimate contact between gel and membrane and to remove any interfering bubbles.

   Poor contact between the gel and membrane will cause a swirled pattern of transferred proteins on the membrane. Some proteins will transfer as soon as the gel is placed on the membrane; repositioning the gel or membrane can result in a smeared or double image on the developed blot.

7. Complete the transfer stack by putting the three remaining sheets of filter paper on top of gel. Roll out bubbles as described above.

   Multiple gels can be transferred using semidry blotting. Simply put a sheet of porous cellophane (Amersham Pharmacia Biotech) or dialysis membrane (Bio-Rad or Sartorius) equilibrated with transfer buffer between each transfer stack (Fig. 6.2.2). Transfer efficiency is dependent on the position of the transfer stack in the blotting unit and for critical applications transferring one gel at a time is recommended. The gel next to the anode tends to be more efficiently transferred when blotting more than one gel at a time.

Transfer proteins from gel to membrane

8. Place top electrode onto transfer stack.

   Most units have safety-interlock features and can only be assembled one way. Consult manufacturer's instructions for details.

   Once assembled, do not move the top electrode. This can shift the transfer stack and move the gel relative to the membrane. Some transfer will occur as soon as the gel contacts the membrane, and any shifting of the transfer stack after assembly will distort the transfer pattern.

9. Carefully connect high-voltage leads to the power supply (see *UNIT 6.1* for safety precautions). Apply constant current to initiate protein transfer. Transfers of 1 hr are generally sufficient.

   In general, do not exceed 0.8 mA/cm^2 of gel area. For a typical minigel (8 × 10 cm) and standard-sized gel (14 × 14 cm) this means ~60 and 200 mA, respectively.

   Monitor the temperature of the transfer unit directly above the gel by touch. The unit should not exceed 45°C. If the outside of the unit is warm, too much current is being applied. Note that units with graphite electrodes are more prone to heating, because graphite has much more resistance to current flow than platinum or steel electrodes.

10. After transfer, turn off power supply and disassemble unit. Remove membrane from transfer stack, marking orientation as in step 12 of Basic Protocol 1. Proceed with staining and immunoprobing (see Basic Protocol 1, steps 13 and 14).

ALTERNATE PROTOCOL 2

BLOTTING OF STAINED GELS

Gels stained with Coomassie blue R250 can be effectively immunoblotted by the following procedure, based on Perides et al. (1986) and Dionisi et al. (1995). Briefly, the stained gel is soaked in a series of solutions designed to increase the solubility of the proteins after staining and fixation. After transfer, the membranes are treated with 45% or 100% methanol to decrease the Coomassie blue bound to the membrane prior to processing for chromogenic development. For chemiluminescent development, removal of the Coomassie blue is generally not needed.

Materials

Destained gel containing proteins of interest
25 mM Tris base/192 mM glycine/1% SDS
25 mM Tris base/192 mM glycine/0.1% SDS

1. Soak destained gel containing proteins of interest in distilled water for 15 min.
2. Equilibrate gel with 25 mM Tris base/192 mM glycine/1% SDS for 1 hr with gentle agitation.
3. Transfer gel to 25 mM Tris base/192 mM glycine/0.1% SDS and equilibrate 30 min with gentle agitation.

   To increase transfer efficiency of larger proteins, the gel should be transferred to the above solution with 6 M urea for an additional 30 min.

4. Proceed with transfer (see Basic Protocol 1, steps 2 to 12).

   For the most efficient transfer and binding to the membrane, the transfer buffer should contain SDS.

5. After transfer, soak membranes for 10 to 30 min in 45% methanol (nitrocellulose) or 100% methanol (nylon or PVDF) to remove the bound Coomassie blue.

   This step is not needed if using chemiluminescent reactions or radiolabeled protein A for immunodevelopment. Destaining of the nitrocellulose membrane is enhanced by adding a small ball of laboratory tissue to the methanol to absorb the Coomassie blue.

6. Proceed with immunoprobing and visual detection of proteins (see Basic Protocols 2 and 3 and Alternate Protocols 3 and 4).

REVERSIBLE STAINING OF TRANSFERRED PROTEINS

SUPPORT PROTOCOL 1

To verify transfer efficiency, nitrocellulose and PVDF membranes can be reversibly stained. This method will not work on nylon membranes.

Additional Materials *(also see Basic Protocol 1)*

Ponceau S solution (see recipe)

Additional reagents and equipment for photographing membranes

1. Following protein transfer to nitrocellulose or PVDF (see Basic Protocol 1 or Alternate Protocol 1), place membrane in Ponceau S solution 5 min at room temperature.
2. Destain 2 min in water. Photograph membrane if required and mark the molecular-weight-standard band locations with indelible ink.
3. Completely destain membrane by soaking an additional 10 min in water.

QUANTITATION OF PROTEIN WITH PONCEAU S

SUPPORT PROTOCOL 2

In addition to qualitatively visualizing proteins on membranes after blotting, Ponceau S provides a convenient method for quantifying the amount of protein in a given lane. By eluting the dye off the strip and reading in a spectrophotometer (A_{525}), an internal control value of protein on a lane is obtained. This value is used to correct for any differences in protein loading from lane to lane. Comparison of the Ponceau S value to the chemiluminescent or chromogenic immunodetection value determined by densitometry provides a straightforward correction for lane-to-lane variation. This method works best for complex mixtures where the immunodetected protein represents a small proportion of the total protein (Klein et al., 1995).

Additional Materials *(also see Basic Protocol 1)*

Spectrophotometer and 2-ml cuvette

1. Following protein transfer to nitrocellulose, PVDF, or nylon (see Basic Protocol 1 or Alternate Protocol 1), stain membrane, photograph, and destain (see Support Protocol 1).

   Membranes should be destained until the background becomes white.

2. Mark lanes with a soft pencil and cut lanes into strips.
3. Place each strip into 7 ml of distilled water for 7 min and remove the resulting solution. If any particulates are visible, centrifuge 30 min at 2000 rpm to remove them.
4. Read A_{525} in a 2-ml cuvette.

   Any variation in gel-to-gel sample loading and blotting efficiency will be reflected in a change in A_{525} of the sample lanes when compared to the control. The change in A_{525} can be calibrated to a known amount of protein loaded on the control lane. This will be a relative value, however, since the transfer out of the gel and binding to the membrane is rarely 100%.

BASIC PROTOCOL 2

IMMUNOPROBING WITH DIRECTLY CONJUGATED SECONDARY ANTIBODY

Immobilized proteins are probed with specific antibodies to identify and quantitate any antigens present. The membrane is immersed in blocking buffer to fill all protein-binding sites with a nonreactive protein or detergent. Next, it is placed in a solution containing the antibody directed against the antigen (primary antibody). The blot is washed and exposed to an enzyme-antibody conjugate directed against the primary antibody (secondary antibody; e.g., goat anti-rabbit IgG). Antigens are identified by chromogenic or luminescent visualization (see Basic Protocol 3 and Alternate Protocol 4) of the antigen/primary antibody/secondary antibody/enzyme complex bound to the membrane. Tween 20 is a common alternative to protein-blocking agents when using nitrocellulose or PVDF filters.

Materials

Membrane with transferred proteins (see Basic Protocol 1 or Alternate Protocol 1)
Blocking buffer appropriate for membrane and detection protocol (see recipe)
Primary antibody specific for protein of interest
TTBS (nitrocellulose or PVDF) or TBS (nylon; see *APPENDIX 2A* for recipes)
Secondary antibody conjugate: horseradish peroxidase (HRPO)–or alkaline phosphatase (AP)–anti-Ig conjugate (Cappel, Vector Labs, Kirkegaard & Perry, or Sigma; dilute as indicated by manufacturer and store frozen in 25-µl aliquots until use)
Heat-sealable plastic bag
Powder-free gloves
Plastic box

1. Place membrane in heat-sealable plastic bag with 5 ml blocking buffer and seal bag. Incubate 30 min to 1 hr at room temperature with agitation on an orbital shaker or rocking platform.

   Usually 5 ml buffer is sufficient for two to three membranes (14 × 14–cm size). If membrane is to be stripped and reprobed (see Support Protocol 3), blocking buffer must contain casein (for AP systems) or nonfat dry milk.

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Dilute primary antibody in blocking buffer.

   Primary antibody dilution is determined empirically but is typically 1/100 to 1/1000 for a polyclonal antibody (Fig. 6.2.3), 1/10 to 1/100 for hybridoma supernatants, and ≥1/1000

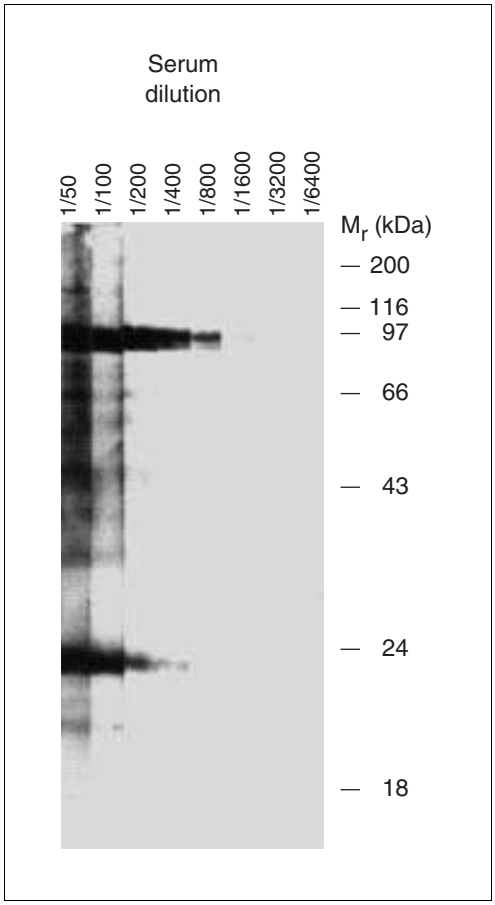

Figure 6.2.3 Serial dilution of primary antibody directed against the 97-kDa catalytic subunit of the plant plasma membrane ATPase. Blot was developed with HRPO-coupled avidin-biotin reagents according to the second alternate protocol and visualized with 4-chloro-1-naphthol (4CN). Note how background improves with dilution.

for murine ascites fluid containing monoclonal antibodies. Ten- to one-hundred-fold higher dilutions can be used with alkaline phosphatase– or luminescence–based detection systems. Both primary and secondary antibody solutions can be used at least twice, but long-term storage (i.e., >2 days at 4°C) is not recommended.

3. Open bag and pour out blocking buffer. Replace with diluted primary antibody and incubate 30 min to 1 hr at room temperature with constant agitation.

   Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14–cm size). Incubation time may vary depending on conjugate used.

   When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from plastic bag with gloved hand. Place in plastic box and wash four times by agitating with 200 ml TTBS (nitrocellulose or PVDF) or TBS (nylon), 10 to 15 min each time.

5. Dilute secondary antibody HRPO- or AP-anti-Ig conjugate in blocking buffer.

   Commercially available enzyme–conjugated secondary antibody is usually diluted 1/200 to 1/2000 prior to use (Harlow and Lane, 1988).

6. Place membrane in new heat-sealable plastic bag, add diluted HRPO- or AP-anti-Ig conjugate, and incubate 30 min to 1 hr at room temperature with constant agitation.

   When using plastic incubation trays, see step 3 annotation for proper antibody solution volumes.

7. Remove membrane from bag and wash as in step 4. Develop according to appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 4).

ALTERNATE PROTOCOL 3

IMMUNOPROBING WITH AVIDIN-BIOTIN COUPLING TO SECONDARY ANTIBODY

The following procedure is based on the Vectastain ABC kit from Vector Labs (see *SUPPLIERS APPENDIX*). It uses an avidin-biotin complex to attach horseradish peroxidase (HRPO) or alkaline phosphatase (AP) to the biotinylated secondary antibody. Avidin-biotin systems are capable of extremely high sensitivity due to the multiple reporter enzymes bound to each secondary antibody. In addition, the detergent Tween 20 is a popular alternative to protein-blocking agents when using nitrocellulose or PVDF filters.

Additional Materials *(also see Basic Protocol 2)*

Blocking buffer appropriate for membrane and detection protocol (see recipe)
TTBS (nitrocellulose or PVDF) *or* TBS (neutral or positively charged nylon; see *APPENDIX 2A* for recipes)
Vectastain ABC (HRPO) or ABC-AP (AP) kit (Vector Labs) containing the following: reagent A (avidin), reagent B (biotinylated HRPO or AP), and biotinylated secondary antibody (request membrane immunodetection protocols when ordering)

1. Equilibrate membrane in appropriate blocking buffer in heat-sealed plastic bag with constant agitation using an orbital shaker or rocking platform. For nitrocellulose and PVDF, incubate 30 to 60 min at room temperature. For nylon, incubate ≥2 hr at 37°C.

   TTBS is well suited for avidin-biotin systems. For nylon, protein-binding agents are recommended. Because nonfat dry milk contains residual biotin, which will interfere with the immunoassay, it must be used in the blocking step only. If membrane is to be stripped and reprobed (see Support Protocol 3), blocking buffer must contain casein (for AP systems) or nonfat dry milk.

   Plastic incubation trays are often used in place of heat-sealable bags, and can be especially useful when processing large numbers of strips in different primary antibody solutions.

2. Prepare primary antibody solution in TTBS (nitrocellulose or PVDF) or TBS (nylon).

   Dilutions of sera containing primary antibody generally range from 1/100 to 1/100,000. This depends in large part on the sensitivity of the detection system. With high-sensitivity avidin-biotin systems, dilutions from 1/1000 to 1/100,000 are common. Higher dilutions can be used with AP- or luminescence-based detection systems. To determine the appropriate concentration of the primary antibody, a dilution series is easily performed with membrane strips. Separate antigens on a preparative gel (i.e., a single large sample well) and immunoblot the entire gel. Cut 2- to 4-mm strips by hand or with a membrane cutter (Schleicher and Schuell; Inotech) and incubate individual strips in a set of serial dilutions of primary antibody. The correct dilution should give low background and high specificity (Fig. 6.2.3).

3. Open bag, remove blocking buffer, and add enough primary antibody solution to cover membrane. Incubate 30 min at room temperature with gentle rocking.

   Usually 5 ml diluted primary antibody solution is sufficient for two to three membranes (14 × 14–cm size). Incubation time may vary depending on conjugate used.

   When using plastic trays, the primary and secondary antibody solution volume should be increased to 25 to 50 ml. For membrane strips, incubation trays with individual slots are recommended. Typically, 0.5 to 1 ml solution/slot is needed.

4. Remove membrane from bag and place in plastic box. Wash membrane three times over a 15-min span in TTBS (nitrocellulose or PVDF) or TBS (nylon). Add enough TTBS or TBS to fully cover the membrane (e.g., 5 to 10 ml/strip or 25 to 50 ml/whole membrane).

5. Prepare biotinylated secondary antibody solution by diluting two drops biotinylated antibody with 50 to 100 ml TTBS (nitrocellulose or PVDF) or TBS (nylon).

   This dilution gives both high sensitivity and enough volume to easily cover a large 14 × 14–cm membrane.

6. Transfer membrane to fresh plastic bag containing secondary antibody solution. Incubate 30 min at room temperature with slow rocking, then wash as in step 4.

   When using plastic incubation trays, see step 3 annotations for proper antibody solution volumes.

7. While membrane is being incubated with secondary antibody, prepare avidin-biotin-HRPO or -AP complex. Mix two drops Vectastain reagent A and two drops reagent B into 10 ml TTBS (nitrocellulose or PVDF) or TBS (nylon). Incubate 30 min at room temperature, then further dilute to 50 ml with TTBS or TBS.

   Diluting the A and B reagents to 50 ml expands the amount of membrane that can be probed without greatly affecting sensitivity. Sodium azide is a peroxidase inhibitor and should not be used as a preservative. Casein, nonfat dry milk, serum, and some grades of BSA may interfere with the formation of the avidin-biotin complex and should not be used in the presence of avidin or biotin reagents (Gillespie and Hudspeth, 1991; Vector Labs).

8. Transfer membrane to avidin-biotin-enzyme solution. Incubate 30 min at room temperature with slow rocking, then wash over a 30-min span as in step 4.

9. Develop membrane according to the appropriate visualization protocol (see Basic Protocol 3 or Alternate Protocol 4).

VISUALIZATION WITH CHROMOGENIC SUBSTRATES

BASIC PROTOCOL 3

Bound antigens are typically visualized with chromogenic substrates. The substrates 4CN, DAB/$NiCl_2$, and TMB are commonly used with horseradish peroxidase (HRPO)–based immunodetection procedures, while BCIP/NBT is recommended for alkaline phosphatase (AP)–based procedures (see Table 6.2.1). After incubation with primary and secondary antibodies, the membrane is placed in the appropriate substrate solution. Protein bands usually appear within a few minutes.

Materials

Membrane with transferred proteins and probed with antibody-enzyme complex (see Basic Protocol 2 or Alternate Protocol 3)
TBS (*APPENDIX 2A*)
Chromogenic visualization solution (Table 6.2.1)

Additional reagents and equipment for gel photography

1. If final membrane wash (see Basic Protocol 2, step 7, or see Alternate Protocol 3, step 8) was performed in TTBS, wash membrane 15 min at room temperature in 50 ml TBS.

   The Tween 20 in TTBS interferes with 4CN development (Bjerrum et al., 1988).

2. Place membrane into chromogenic visualization solution. Bands should appear in 10 to 30 min.

3. Terminate reaction by washing membrane in distilled water. Air dry and photograph for a permanent record.

Table 6.2.1 Chromogenic and Luminescent Visualization Systems[a]

System	Reagent[b]	Reaction/Detection	Comments[c]
Chromogenic			
HRPO-based	4CN	Oxidized products form purple precipitate	Not very sensitive (Tween 20 inhibits reaction); fades rapidly upon exposure to light
	DAB/$NiCl_2$[d]	Forms dark brown precipitate	More sensitive than 4CN but potentially carcinogenic; resulting membrane easily scanned
	TMB[e]	Forms dark purple stain	More stable, less toxic than DAB/$NiCl_2$; may be somewhat more sensitive[e]; can be used with all membrane types; kits available from Kirkegaard & Perry, TSI, Moss, and Vector Labs
AP-based	BCIP/NBT	BCIP hydrolysis produces indigo precipitate after oxidation with NBT; reduced NBT precipitates; dark blue-gray stain results	More sensitive and reliable than other AP-precipitating substrates; note that phosphate inhibits AP activity
Luminescent			
HRPO-based	Luminol/H_2O_2/ *p*-iodophenol	Oxidized luminol substrate gives off blue light; *p*-iodophenol increases light output	Very convenient, sensitive system; reaction detected within a few seconds to 1 hr
AP-based	Substituted 1,2-dioxetane-phosphates (e.g., AMPPD, CSPD, Lumigen-PPD, Lumi-Phos 530[f])	Dephosphorylated substrate gives off light	Protocol described gives reasonable sensitivity on all membrane types; consult instructions of reagent manufacturer for maximum sensitivity and minimum background (see Troubleshooting)

[a]Abbreviations: AMPPD or Lumigen-PPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-tricyclo[3.3.1.1^{3,7}] decan}-4-yl)phenyl phosphate; AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 4CN, 4-chloro-1-napthol; CSPD, AMPPD with substituted chlorine moiety on adamantine ring; DAB, 3,3′-diaminobenzidine; HRPO, horseradish peroxidase; NBT, nitroblue tetrazolium; TMB, 3,3′,5,5′-tetramethylbenzidine.

[b]Recipes and suppliers are listed in Reagents and Solutions except for TMP, for which use of a kit is recommended.

[c]See Commentary for further details.

[d]DAB/$NiCl_2$ can be used without the nickel enhancement, but it is much less sensitive.

[e]McKimm-Breschkin (1990) reported that if nitrocellulose filters are first treated with 1% dextran sulfate for 10 min in 10 mM citrate-EDTA (pH 5.0), TMB precipitates onto the membrane with a sensitivity much greater than 4CN or DAB, and equal to or better than that of BCIP/NBT.

[f]Lumi-Phos 530 contains dioxetane phosphate, $MgCl_2$, CTAB (cetyltrimethylammonium bromide), and fluorescent enhancer in a pH 9.6 buffer.

ALTERNATE PROTOCOL 4

VISUALIZATION WITH LUMINESCENT SUBSTRATES

Antigens can also be visualized with luminescent substrates. Detection with light offers both speed and enhanced sensitivity over chromogenic and radioisotopic procedures. After the final wash, the blot is immersed in a substrate solution containing luminol for horseradish peroxidase (HRPO) systems or dioxetane phosphate for alkaline phosphatase (AP) systems, sealed in thin plastic wrap, and placed firmly against film. Exposures range from a few seconds to several hours, although typically strong signals appear within a few seconds or minutes.

Additional Materials *(also see Basic Protocol 3)*

Luminescent substrate buffer: 50 mM Tris·Cl, pH 7.5 (HRPO; *APPENDIX 2A*) *or* dioxetane phosphate substrate buffer (alkaline phosphatase; see recipe)
Nitro-Block solution (AP reactions only): 5% (v/v) Nitro-Block (Tropix) in dioxetane phosphate substrate buffer, prepared just before use
Luminescent visualization solution (Table 6.2.1)
Clear plastic wrap

Additional reagents and equipment for autoradiography (*UNIT 6.3*)

NOTE: See Troubleshooting section for suggestions concerning optimization of this protocol, particularly when employing AP-based systems.

1. Equilibrate membrane in two 15-min washes with 50 ml substrate buffer.

   For blots of whole gels, use 50 ml substrate buffer; for strips, use 5 to 10 ml/strip.

2. *For AP reactions using nitrocellulose or PVDF membranes:* Incubate 5 min in Nitro-Block solution, followed by 5 min in substrate buffer (volumes as in step 1).

   Nitro-Block enhances light output from the dioxetane substrate in reactions using AMPPD, CSPD, or Lumigen-PPD concentrate. It is required for nitrocellulose and recommended for PVDF membranes. It is not needed for Lumi-Phos 530, AP reactions on nylon membranes, or HRPO-based reactions on any type of membrane. Lumi-Phos 530 is not recommended for nitrocellulose membranes.

3. Transfer membrane to visualization solution. Soak 30 sec (HRPO reactions) to 5 min (AP reactions; volumes as in step 1).

   Alternatively, lay out a square of plastic wrap and pipet 1 to 2 ml visualization solution into the middle. Place membrane on the plastic so that the visualization solution spreads out evenly from edge to edge. Fold wrap back onto membrane, seal, and proceed to step 5.

4. Remove membrane, drain, and place face down on a sheet of clear plastic wrap. Fold wrap back onto membrane to form a liquid-tight enclosure.

   To ensure an optimal image, only one layer of plastic should be between the membrane and film. Sealable bags are an effective alternative. Moisture must not come in contact with the X-ray film.

5. In a darkroom, place membrane face down onto film.

   Do this quickly and do not reposition; a double image will be formed if the membrane is moved while in contact with the film. A blurred image is usually caused by poor contact between membrane and film; use a film cassette that ensures a tight fit.

6. Expose film for a few seconds to several hours.

   Typically, immunoblots produce very strong signals within a few seconds or minutes. However, weak signals may require several hours to an overnight exposure. If no image is detected, expose film 30 min to 1 hr, and if needed, overnight (see Troubleshooting).

7. If desired, wash membrane in two 15-min washes of 50 ml TBS and process for chromogenic development (see Basic Protocol 3).

   Chemiluminescent and chromogenic immunoblotting can be easily combined on a single blot to provide a permanent visual marker of a known protein. First probe membrane with the chemiluminescent reactions to record on film. If stripping and reprobing is needed, then process by wetting and NaOH treatment (see Support Protocol 3). For the last reaction, use chromogenic development to produce a permanent visual record of the blot. Alternatively, once the film record of the chemiluminescent blot is recorded, the blot can be rinsed briefly with distilled water and placed in the appropriate chromogenic solution for chromogenic development of the blot. This results in a permanent reference stain on the blot for comparison to the more easily scanned and quantitated film record.

SUPPORT PROTOCOL 3

STRIPPING AND REUSING MEMBRANES

This stripping procedure works with blotted membranes from one- and two-dimensional gels as well as with proteins blotted from previously stained gels (Suck and Krupinska, 1996). Reprobing PVDF membranes that have been developed with chemiluminescent reagents is simple and straightforward. All residual antibodies are removed from the membrane by first rewetting it in water and then briefly treating it with NaOH. Although repeated reprobing can lead to loss of signal, up to five reprobings generally are feasible. The blot should have been previously blocked with 5% nonfat dry milk prior to treatment.

Materials

0.2 M NaOH

1. Wash blot 5 min in distilled water.

   In order to effectively reprobe the membranes, casein (for AP systems) or nonfat dry milk must be used as the blocking agent. Chromogenic development leaves a permanent stain on the membrane that is difficult to remove, and should not be used when reprobing. The stain can interfere with subsequent analysis if reactive bands from sequential immunostainings are close together.

2. Transfer to 0.2 M NaOH and wash 5 min.
3. Wash blot 5 min in distilled water.
4. Proceed with immunoprobing procedure (see Basic Protocol 2 and Alternate Protocol 3).

   Casein or nonfat dry milk is recommended as blocking agent when reprobing membranes.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**. For selection of appropriate chromogenic or luminescent solutions, and for definition of abbreviations, see Table 6.2.1.*

Alkaline phosphate substrate buffer

100 mM Tris·Cl, pH 9.5
100 mM NaCl
5 mM $MgCl_2$

BCIP/NBT visualization solution

Mix 33 µl NBT stock (100 mg NBT in 2 ml at 70% DMF, stored <1 year at 4°C) and 5 ml alkaline phosphate substrate buffer (see recipe). Add 17 µl BCIP stock (100 mg BCIP in 2 ml of 100% DMF, stored <1 year at 4°C) and mix. Stable 1 hr at room temperature.

Recipe is from Harlow and Lane (1988). Alternatively, BCIP/NBT substrates may be purchased from Sigma, Kirkegaard & Perry, Moss, and Vector Labs.

Blocking buffer

Colorimetric detection:

For nitrocellulose and PVDF: 0.1% (v/v) Tween 20 in TBS (TTBS; *APPENDIX 2A*).

For neutral and positively charged nylon: Tris-buffered saline (TBS; *APPENDIX 2A*) containing 10% (w/v) nonfat dry milk. Prepare just before use.

TTBS can be stored ≤1 week at 4°C.

Luminescence detection:

For nitrocellulose, PVDF, and neutral nylon (e.g., Pall Biodyne A): 0.2% casein (e.g., Hammarsten grade or I-Block; Tropix) in TTBS (*APPENDIX 2A*). Prepare just before use.

continued

For positively charged nylon: 6% (w/v) casein/1% (v/v) polyvinyl pyrrolidone (PVP) in TTBS (*APPENDIX 2A*). With constant mixing, add casein and PVP to warm (65°C) TTBS. Stir for 5 min. Cool before use. Prepare just before use.

4CN visualization solution

Mix 20 ml ice-cold methanol with 60 mg 4CN. Separately mix 60 µl of 30% H_2O_2 with 100 ml TBS (*APPENDIX 2A*) at room temperature. Rapidly mix the two solutions and use immediately.

DAB/$NiCl_2$ visualization solution

5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
100 µl DAB stock (40 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
25 µl $NiCl_2$ stock (80 mg/ml in H_2O, stored in 100-µl aliquots at −20°C)
15 µl 3% H_2O_2
Mix just before use

CAUTION: *Handle DAB carefully, wearing gloves and mask; it is a carcinogen.*

Suppliers of peroxidase substrates are Sigma, Kirkegaard & Perry, Moss, and Vector Labs.

Dioxetane phosphate substrate buffer

1 mM $MgCl_2$
0.1 M diethanolamine
0.02% sodium azide (optional)
Adjust to pH 10 with HCl and use fresh

Traditionally, the AMPPD substrate buffer has been a solution containing 1 mM $MgCl_2$ and 50 mM sodium carbonate/bicarbonate, pH 9.6 (Gillespie and Hudspeth, 1991). The use of diethanolamine results in better light output (Tropix Western Light instructions).

Alternatively, 100 mM Tris·Cl (pH 9.5)/100 mM NaCl/5 mM $MgCl_2$ can be used (Sandhu et al., 1991).

Dioxetane phosphate visualization solution

Prepare 0.1 mg/ml AMPPD or CSPD (Tropix) *or* Lumigen-PPD (Lumigen; see Table 6.2.1) substrate in dioxetane phosphate substrate buffer (see recipe). Prepare just before use. Lumi-Phos 530 (Boehringer Mannheim or Lumigen) is a ready-to-use solution and can be applied directly to the membrane.

This concentration of AMPPD substrate (240 µM) is the minimum recommended by Tropix Western Light. Ten-fold lower concentrations can be used but require longer exposures.

Luminol visualization solution

0.5 ml 10× luminol stock [40 mg luminol (Sigma) in 10 ml DMSO]
0.5 ml 10× *p*-iodophenol stock [optional; 10 mg (Aldrich) in 10 ml DMSO]
2.5 ml 100 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
25 µl 3% H_2O_2
H_2O to 5 ml
Prepare just before use

Recipe is from Schneppenheim et al. (1991). Premixed luminol substrate mix (Mast Immunosystems; Amersham ECL; Du Pont NEN Renaissance; Kirkegaard & Perry LumiGLO) may also be used. p-iodophenol is an optional enhancing agent that increases light output. Luminol and p-iodophenol stocks can be stored for ≤6 months at −20°C.

Ponceau S solution

Dissolve 0.5 g Ponceau S in 1 ml glacial acetic acid. Bring to 100 ml with water. Prepare just before use.

Transfer buffer

Add 18.2 g Tris base and 86.5 g glycine to 4 liters of water. Add 1200 ml methanol and bring to 6 liters with water. The pH of the solution is ~8.3 to 8.4. For use with PVDF filters, decrease methanol concentration to 15%; for nylon filters, omit methanol altogether.

CAPS transfer buffer can also be used. Add 2.21 g cyclohexylaminopropane sulfonic acid (CAPS; free acid), 0.5 g DTT, 150 ml methanol, and water to 1 liter. Adjust to pH 10.5 with NaOH and chill to 4°C. For proteins >60 kDa, reduce methanol content to 1% (Moos, 1992).

COMMENTARY

Background Information

Immunoprecipitation has been widely used to visualize the antigens recognized by various antibodies, both polyclonal and monoclonal (*UNIT 7.2*). However, there are several problems inherent with this method, including the requirement for radiolabeling of antigen, co-precipitation of tightly associated macromolecules, occasional difficulty in obtaining precipitating antibodies, and insolubility of various antigens (Talbot et al., 1984).

To circumvent these problems, electroblotting (Towbin et al., 1979)—subsequently popularized as western blotting or immunoblotting (Burnette, 1981)—was conceived. Immunoblotting is a rapid and sensitive assay for the detection and characterization of proteins that works by exploiting the specificity inherent in antigen-antibody recognition. It involves the solubilization and electrophoretic separation of proteins, glycoproteins, or lipopolysaccharides by SDS-PAGE (*UNIT 6.1*) or urea-PAGE, followed by quantitative transfer and irreversible binding to nitrocellulose, PVDF, or nylon. This technique has been useful in identifying specific antigens recognized by polyclonal or monoclonal antibodies and is highly sensitive (1 ng of antigen can be detected).

Electroblotting of previously stained gels is a convenient way to visualize and document the gel prior to immunoblotting. Transfer efficiencies at all molecular weights will be lower with fixed and stained gels. This is particularly true of proteins >50 kDa (Perides et al., 1986). The additional incubation in 6 M urea will significantly increase transfer efficiency of all proteins and is required for proteins >50 kDa.

Ponceau S staining provides an easy method for calibrating and quantitating the amount of material on a nitrocellulose or PVDF blot. An alternative to this method is to use an internal protein control with a separate antibody probe, but these tend to be expensive and time-consuming to use. Other applications for Ponceau S calibration include monitoring transfer efficiency under varied conditions for optimization of tank and semidry blotting.

Immunoblotted proteins can be detected by chromogenic or luminescent assays (see Table 6.2.1 for a description of the reagents available for each system, their reactions, and a comparison of their advantages and disadvantages). Luminescent detection methods offer several advantages over traditional chromogenic procedures. In general, luminescent substrates increase the sensitivity of both HRPO and phosphatase systems without the need for radioisotopes. Substrates for the latter have only recently been applied to protein blotting (see Gillespie and Hudspeth, 1991; Sandhu et al., 1991; Bronstein et al., 1992). Luminescent detection can be completed in as little as a few seconds; exposures rarely go more than 1 hr. Depending on the system, the luminescence can last for 3 days, permitting multiple exposures of the same blot. Furthermore, the signal is detected by film, and varying the exposure can result in more or less sensitivity. Luminescent blots can be easily erased and reprobed because the reaction products are soluble and do not deposit on the membrane (see below). Compared to chromogenic development, the luminescent image recorded on film is easier to photograph and to quantitate by densitometry.

Alkaline phosphatase–based luminescent protocols that achieve maximum sensitivity with minimum background can be complex, and the manufacturer's instructions should be consulted (see Reagents and Solutions). The procedure described in Alternate Protocol 4 gives reasonable sensitivity on nitrocellulose, PVDF, and nylon membranes with a minimum of steps.

Critical Parameters

First and foremost, the antibody being used should recognize denatured antigen. Nonspecific binding of antibodies can occur, so control antigens and antibodies should always be run in parallel. Time of transfer and primary anti-

body and conjugate dilutions should always be optimized.

A variety of agents are currently used to block binding sites on the membrane after blotting (Harlow and Lane, 1988). These include Tween 20, PVP, nonfat dry milk, casein, BSA, and serum. A 0.1% (v/v) solution of Tween 20 in TBS (TTBS), a convenient alternative to protein-based blocking agents, is recommended for chromogenic development of nitrocellulose and PVDF membranes (Blake et al., 1984). In contrast to dry milk/TBS blocking solution (BLOTTO), TTBS is stable and has a long shelf life at 4°C. Furthermore, TTBS generally produces a clean background and permits subsequent staining with India ink. However, even with the application of such standard blocking procedures as 5% to 10% milk protein or 0.05% to 0.1% Tween 20, background can still be a significant problem. If this happens, using a blocking protein (e.g., goat, horse, or rabbit normal serum) from the same species as the primary antibody can reduce the background, presumably by reducing cross-reactivity between the primary antibodies and the blocking agent. Combinations of blocking agents can also be effective. Thus, 0.1% human serum albumin (HSA) and 0.05% Tween 20 in TBS is recommended when probing Immobilon-P membranes with human serum (Craig et al., 1993). However, this can also lead to overall loss of antigen signal, requiring a ten-fold increase in the primary antibody (serum) concentration to achieve an adequate background free antigen signal.

When using chemiluminescent detection for immunoblotting, high background frequently occurs, particularly for strong signals (Pampori et al., 1995). Several methods are available for reducing the background from chemiluminescent reactions. These include changing the type and concentration of blocking agents (see above), optimizing antibody concentrations, letting the reaction proceed for several minutes before exposing to film, or simply limiting the exposure time of the film on the blot. These procedures are not always successful, however, and can lead to inconsistent results. An alternative approach is to reduce the concentration of reagents ten-fold. This effectively removes the background and has a number of advantages which include lower cost, increased signal-to-noise ratio, and reduced detection of cross-reacting species.

Two types of nylon membrane are used for western transfer—neutral (e.g., Pall Biodyne A) and positively charged (e.g., Pall Biodyne B). Although the positively charged membranes have very good protein-binding characteristics, they tend to give a higher background. These membranes remain positively charged from pH 3 to pH 10. Neutral nylon membranes are also charged, having a mix of amino and carboxyl groups that give an isoelectric point of 6.5. Because of their high binding capacity, positively charged membranes are popular for protein applications using luminescence.

Nylon membranes require more stringent blocking steps. Here 10% nonfat dry milk in TBS is recommended for chromogenic development. During luminescence development, however, background is a more significant problem. Compared to dry milk, purified casein has minimal endogenous alkaline phosphatase activity (AP activity leads to high background) and is therefore recommended as a blocking agent for nitrocellulose, PVDF, and nylon membranes. Positively charged nylon requires much more stringent blocking with 6% (w/v) casein and 1% (v/v) polyvinylpyrrolidone (PVP). Because nonfat dry milk and casein may contain biotin that will interfere with avidin-biotin reactions, subsequent steps are done without protein-blocking agents when using these systems. If background is a problem, highly purified casein (0.2% to 6%) added to the antibody incubation buffers may help.

If reprobing is desired, blots can be air dried and stored at 4°C for 3 months after chemiluminescence detection. After drying, store in a sealed freezer bag until use. Repeated probing will lead to a gradual loss of signal and increased background. However, this will depend in part on the properties of the sample.

If the primary procedure is problematic due to loss of sensitivity or an increase in the background, then two possible alternative procedures for stripping membranes are recommended. The first uses 2-mercaptoethanol and SDS (Kaufmann et al., 1987; Tesfaigzi et al., 1994). Briefly, the membranes are incubated in 2% SDS/100 mM Tris·Cl, pH 7.4/100 mM 2-mercaptoethanol for 30 min at 70°C, effectively removing primary and secondary antibodies. As with the primary procedure recommended above, the repeated probing should be done with caution due to the potential loss of detection signal, and 5% nonfat dry milk is required as a blocking agent. The milk blocking agent facilitates antibody removal from the blot (Kaufmann et al., 1987). The second uses guanidine·HCl. For nylon and PVDF membranes (do *not* use with nitrocellulose), incubate the immunoblot in 7 M guanidine·HCl for

10 min at room temperature. (The short wash time is critical, as guanidine·HCl is a very strong denaturant, so do not leave the filter in this solution >15 min.) Pour off excess guanidine·HCl and then rinse the membrane several times in 1× TTBS. Reblock the membrane and proceed with the standard immunoblotting procedure. Membranes stripped using this procedure can generally be reused three or four times.

Troubleshooting

There are several problems associated with immunoblotting. The antigen is solubilized and electrophoresed in the presence of denaturing agents (e.g., SDS or urea), and some antibodies may not recognize the denatured form of the antigen transferred to the membrane. The results observed may be entirely dependent on the denaturation and transfer system used. For example, zwitterionic detergents have been shown to restore the antigenicity of outer membrane proteins in immunoblotting (Mandrell and Zollinger, 1984). Gel electrophoresis under nondenaturing conditions can also be performed.

Other potential problems include high background, nonspecific or weak cross-reactivity of antibodies, poor protein transfer or membrane binding efficiency, and insufficient sensitivity. For an extensive survey and discussion of immunoblotting problems and artifacts, see Bjerrum et al. (1988).

If no transfer of protein has occurred, check the power supply and electroblot apparatus to make sure that the proper electrical connections were made and that power was delivered during transfer. In addition, check that the correct orientation of filter and gel relative to the anode and cathode electrodes was used.

If the transfer efficiency using the tank system appears to be low, increase the transfer time or power. Cooling (using the unit's built-in cooling cores) is generally required for transfers >1 hr. At no time should the buffer temperature go above 45°C. Prolonged transfers (>1 hr) are not possible in semidry transfer units due to rapid buffer depletion.

Alternatively, the transfer buffer can be modified to increase efficiency. Adding SDS at a concentration of 0.1% to the transfer buffer improves the transfer of all proteins out of the gel, particularly those above 60 to 90 kD in size. Lowering the concentration of methanol will also improve the recovery of proteins from the gel. These procedures are tradeoffs. Methanol improves the binding of proteins to PVDF and nitrocellulose, but at the same time hinders transfer. With SDS present, transfer efficiency is improved, but the SDS can interfere with protein binding to the membrane. Nylon and PVDF membranes are particularly sensitive to SDS interference. If needed, 0.01% to 0.02% SDS may be used in PVDF membrane transfer buffers (Millipore, 1990). SDS and methanol should not be used in the transfer buffer for nylon (Peluso and Rosenberg, 1987).

Gel cross-linking and thickness also have a profound effect on the transfer efficiency. In general, 0.5- to 0.75-mm-thick gels will transfer much more efficiently than thicker gels (e.g., 1.5 mm thick). Gels with a higher acrylamide percentage will also transfer less efficiently. Proteins can be particularly difficult to transfer from gradient gels, and a combination of longer transfer times, thin gels, and the addition of SDS to the transfer buffer may be needed.

If the protein bands are diffuse, check the transfer cassette. The gel must be held firmly against the membrane during transfer. If the transfer sandwich is loose in the cassette, add another thin sponge or more blotter paper to both sides.

Occasionally, a grid pattern will be apparent on the membrane after tank transfer. This is caused by having either the gel or the membrane too close to the sides of the cassette. Correct this by adding more layers of filter paper to diffuse the current flowing through the gel and membrane. Use a thinner sponge and more filter paper if necessary.

If air bubbles are trapped between the filter and the gel, they will appear as clear white areas on the filter after blotting and staining. Take extra care to make sure that all bubbles are removed.

Insufficient blocking or nonspecific binding of the primary or secondary antibody will cause a high background stain. A control using preimmune serum or only the secondary antibody will determine if these problems are due to the primary antibody. Try switching to another blocking agent; protein blocking agents may weakly cross-react. Lowering the concentration of primary antibody should decrease background and improve specificity (Fig. 6.2.3).

Due to the nature of light and the method of detection, certain precautions are warranted when using luminescent visualization (e.g., Harper and Murphy, 1991). Very strong signals can overshadow nearby weaker signals on the membrane. Because light will pipe through the membrane and the surrounding plastic wrap, overexposure will produce a broad diffuse im-

age on the film. The signal can also saturate the film, exposing the film to a point whereby increased exposure will not cause a linear increase in the density of the image on the film.

With the alkaline phosphatase substrate AMPPD, nitrocellulose, PVDF, and nylon membranes require 2, 4, and 8 to 12 hr, respectively, to reach maximum light emission. In addition, PVDF is reported to give a stronger signal than nitrocellulose (Tropix Western Light instructions). Positively charged nylon requires special blocking procedures to minimize background (Gillespie and Hudspeth, 1991). These procedures include using a blocking and primary antibody solution containing 6% casein, 1% polyvinylpyrrolidone-40 (PVP-40), 3 mM NaN_3, 10 mM EDTA, and PBS, pH 6.8. Prior to use, the casein must be heated to 65°C to reduce alkaline phosphatase activity in the casein itself. In addition, maximum sensitivity has been observed when free biotin or biotinylated proteins are removed by pretreating the casein with avidin-agarose (Sigma).

Anticipated Results

Immunoblotting should result in the detection of one or more bands. Although antibodies directed against a single protein should produce a single band, degradation of the sample (e.g., via endogenous proteolytic activity) may cause visualization of multiple bands of slightly different size. Multimers will also form spontaneously, causing higher-molecular-weight bands on the blot. If simultaneously testing multiple antibodies directed against a complex protein mixture (e.g., using patient sera against SDS-PAGE-separated viral proteins in AIDS western blot test), multiple bands will be visualized. Typically, picogram to nanogram sensitivities are common in protein blotting and immunodetection procedures.

Time Considerations

The entire immunoblotting procedure can be completed in 1 to 2 days, depending on transfer time and type of gel. Gel electrophoresis requires 4 to 6 hr on a regular gel and 1 hr on a minigel. Transfer time can be 1 hr (high-power transfer) to overnight. Blocking, conjugate incubation, and washing each take 30 min to 1 hr. Finally, substrate incubation requires 10 to 30 min (chromogen) and a few seconds to several hours (luminescence).

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Detection and Quantitation of Radiolabeled Proteins in Gels and Blots

This unit presents procedures for visualizing and quantitating radiolabeled proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 6.1*) or affixed to filter membranes. Autoradiography (see Basic Protocol) is the most common method by which this is accomplished, and X-ray film is the traditional recording medium. The use of autoradiography with gels requires that the gel be dried prior to being placed in contact with the film (see Support Protocol 1). The decay of radioactive materials within the dried gel or filter leaves an image on the film that reflects its distribution in the sample. Film images can be quantified by densitometry (see Support Protocol 4) to obtain a relative measure of the amount of radioactivity in the sample.

The use of X-ray films for autoradiography, however, suffers from two drawbacks: lack of sensitivity and a limited linear range over which the image density reflects the amount of radioactivity. Lack of sensitivity can be overcome by fluorography (see Alternate Protocol 1) or by the use of intensifying screens (see Support Protocol 2), both of which enhance the radioactive signal. Ensuring that the exposure is within a linear range requires careful controls; film is often preflashed (see Support Protocol 3) to increase the linear measurement range for weakly radioactive samples, and it is important to ensure that the film not be saturated to attain strong radioactive signals. Sensitivity and linear ranges of measurement can be greatly extended by using a phosphor imaging system (see Alternate Protocol 2). Phosphor imaging also makes it much faster and easier to quantify radioactive samples.

To enhance radioactive signals, solid-state scintillation is frequently employed to convert the energy released by radioactive molecules to visible light. This is accomplished in several different ways. In fluorography (see Alternate Protocol 1) organic scintillants are incorporated into the sample to increase the proportion of emitted energy detected from low-energy β particles (e.g., from ^{3}H, ^{14}C, and ^{35}S). Another method uses high-density, fluorescent "intensifying screens" (see Support Protocol 2), which are placed next to the sample and used to capture the excess energy of γ rays (e.g., those produced by ^{125}I) and high-energy β particles (e.g., from ^{32}P).

CAUTION: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in appropriately designated areas, following the guidelines provided by your local radiation safety officer (also see *APPENDIX 1D*).

AUTORADIOGRAPHY

BASIC PROTOCOL

Autoradiography uses X-ray film to visualize and quantitate radioactive molecules that have been electrophoresed through agarose or polyacrylamide gels (*UNIT 6.1*), hybridized to filters (e.g., immunoblots; *UNIT 6.2*), or chromatographed through paper or thin-layer plates. A photon of light or the β particles and γ rays released from radioactive molecules "activate" silver bromide crystals on the film emulsion. This renders them capable of being reduced through the developing process to form silver metal (a "grain"). The silver grains on the film form the image.

The choice of film is critical for autoradiography. Double-coated films (e.g., Kodak X-Omat AR and Fuji RX) contain two emulsion layers on either side of a polyester support and are most commonly used for autoradiography (Laskey and Mills, 1977). Double-coated films are ideal for detecting the high-energy β particles emitted by ^{32}P and ^{125}I,

Contributed by Daniel Voytas and Ning Ke
Current Protocols in Cell Biology (1998) 6.3.1-6.3.10
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since they can penetrate the polyester support and expose both emulsion layers. These films are normally used with calcium tungstate ($CaWO_4$) intensifying screens at reduced temperature (−70°C); they are highly sensitive to the blue light emitted by these screens. The green-light-sensitive BioMax MS film (Kodak) is a double-coated film spectrally matched to the blue- and green-light-emitting BioMax MS intensifying screen. The BioMax MS film/BioMax MS intensifying screen system normally gives greatest sensitivity to ^{32}P (four times greater than that of X-Omat AR film with $CaWO_4$ screens).

Single-coated films, containing one emulsion layer (e.g., Kodak BioMax MR), are optimized for direct-exposure techniques with medium-energy radioisotopes (e.g., ^{14}C, ^{35}S, and ^{33}P, but not ^{3}H). The majority of the β particles emitted by these isotopes cannot pass through the polyester support of double-coated films, and therefore the emulsion layer on the other side of the film is useless. Even though direct exposure with single-coated films gives better clarity for medium-energy isotopes, single-coated films often require longer exposure times. Fluorography, therefore, is often used to enhance sensitivity. The blue-light-sensitive double-coated X-Omat AR film is generally used for fluorography with 2,5-diphenyloxazole (PPO; which emits at 388 nm), sodium salicylate (which emits at 420 nm), and commercial fluorographic solutions and sprays (e.g., from Amersham and DuPont NEN; which emit light in the blue end of the spectrum).

Materials

Fixed and dried gel (see Support Protocol 1) or filter (e.g., from immunoblotting; *UNIT 6.2*)
Developer: Kodak developer and replenisher, prepared according to the manufacturer's instructions, 18° to 20°C
Fixer: Kodak fixer and replenisher, prepared according to the manufacturer's instructions, 18° to 20°C
Metal film cassette or paper film cassette with particle-board supports and metal binder clips
Plastic wrap (e.g., Saran Wrap)
X-ray film
Trays to hold film processing solutions
Clips for hanging film

1. In a darkroom illuminated with a safelight, place the sample (e.g., dried gel or filter) in the film cassette. Cover the sample with plastic wrap to prevent it from sticking to the film and contaminating the cassette with radioactivity.

   The safelight should be a bulb of <15 W that is equipped with a Kodak GBX-2 red filter (or equivalent).

   Fluorescent glow-in-the-dark ink (available at craft stores) is a convenient way to mark samples exposed to film. The ink can be spread on adhesive labels, which in turn are placed on the plastic wrap around the edge of the sample. If exposed to light prior to autoradiography, the ink will fluoresce and expose the film, making it possible to orient the film image on the dried gel after developing.

2. Place a sheet of X-ray film on top of the sample, then close and secure the film cassette (see Fig. 6.3.1).

   If preflashed film is used for direct exposure (see Support Protocol 3), the exposed side should face the sample. Preflashed film should be used if sample is weakly radioactive or if quantitation of the radioactivity is desired. For single-coated film, the emulsion layer should face the sample.

   If a paper cassette is used, particle-board supports the same size as the cassette should be placed on either side and secured with the metal binder clips. This will ensure that the sample and film are held in contact and do not shift during exposure.

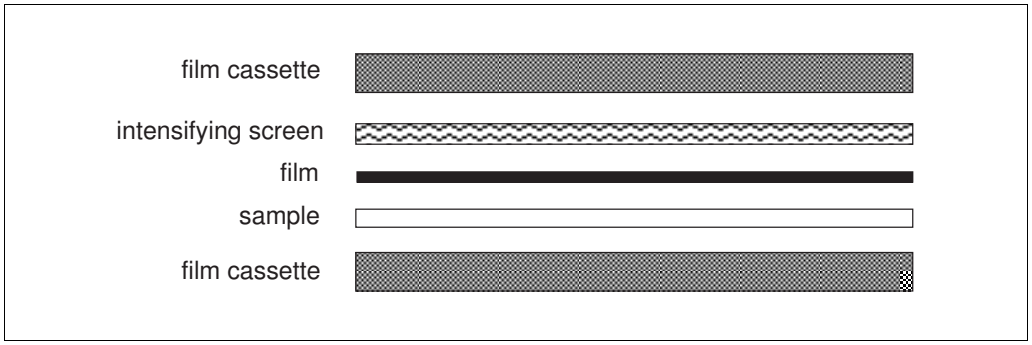

Figure 6.3.1 Autoradiography setup: intensifying screen, film, and sample in film cassette.

3. Expose the film for the desired length of time and at the appropriate temperature.

   Time of exposure will depend on the strength of the radioactivity in the sample and, in most cases, will have to be determined empirically by making multiple exposures for different lengths of time. To help estimate exposure time, a Geiger counter can often be used to detect the relative amount of radioactivity in the sample. With experience, this can help alleviate the trial and error often associated with obtaining the optimum exposure. Time of exposure and use of internal controls are particularly important if quantitative comparisons between experiments are desired.

4. After exposure, return cassette to the darkroom and remove film for developing.

   If the film was exposed at −70°C, allow the cassette to come to room temperature before developing. This will avoid static discharge, which can cause black dots or stripes on the autoradiogram.

   Automated film developers are also available and can be used to develop the film.

5. Immerse the film for 5 min in 18° to 20°C developer, then wash 1 min in running water at room temperature.

   Shorter periods of time in developer will yield a lighter image. The amount of time in developer, therefore, can be used to roughly control intensity of the image.

6. Immerse the film for 5 min in 18° to 20°C fixer, then wash for 15 min in running water.

7. Hang the film to dry.

   The orientation of the film with respect to the gel can be determined by the images of the fluorescent markers.

FIXING AND DRYING GELS FOR AUTORADIOGRAPHY

SUPPORT PROTOCOL 1

SDS-PAGE gels containing radiolabeled proteins should be fixed and dried before exposure to film. This will prevent the gel from sticking to the film, improve the sharpness of the image, and increase sensitivity slightly. However, if the specific activity of the sample is high or the detection method is sensitive (e.g., where a phosphor imager is used; see Alternate Protocol 2), then fixing and drying the gel may not be necessary. Gel dryers are available from a number of manufacturers (e.g., Bio-Rad), most of which use heat and a vacuum to accelerate the drying process.

Materials

Gel from SDS-PAGE (*UNIT 6.1*)
Fixing solution: 10% (v/v) glacial acetic acid/20% (v/v) methanol in H_2O
Alternative fixing solution (for gels with ≥15% acrylamide or thicker than 1.5 mm): 3% (v/v) glycerol/10% (v/v) glacial acetic acid/20% (v/v) methanol in H_2O

Glass dish
Rotary shaker
Filter paper (Whatman 3MM) in sheets at least 1 to 2 cm larger than gel
Plastic wrap (e.g., Saran Wrap)
Gel dryer with vacuum pump

1. After electrophoresis, remove the gel and the supporting glass plates from the electrophoresis apparatus and place in a glass dish. Carefully remove the upper glass plate by gently prying apart the corners with a metal spatula. Make a notch in the upper right hand corner of the gel for orientation.

   Since the gel contains radiolabeled proteins, be sure to follow the necessary guidelines for handling radioactivity. Everything that comes in contact with the gel is potentially radioactive.

2a. *For gels with <15% acrylamide and <1.5 mm thick:* Place the glass dish in a fume hood and pour enough fixing solution into the dish to cover the gel. Place the dish on a rotary shaker and gently rotate until 5 min after all of the blue color from the bromphenol blue in the sample buffer (if used) has disappeared (~30 min total).

   The bromphenol blue typically used in SDS-PAGE sample buffer will turn yellow as the acidic fixing solution diffuses into the gel.

   During fixing, the gel will typically slide off the lower glass plate, which can be removed.

2b. *For gels with ≥15% acrylamide or >1.5 mm thick:* Fix gel as in step 2a, but soak 1 hr in alternative fixing solution.

   The glycerol in the alternative fixing solution should help prevent cracking during drying.

3. Pour off the fixing solution and rinse the gel for a few minutes with deionized water.

   CAUTION: *Remember that solutions that come in contact with the gel are potentially radioactive.*

4. Carefully pour off the water and position the gel in the center of the glass dish. Be sure that any excess water is drained. Place a sheet of Whatman 3MM filter paper, at least 1 to 2 cm larger than the gel, over the gel.

   The gel will stick to the filter paper, which will allow it to be lifted and turned over with the gel side facing up.

5. Cover gel with plastic wrap. Smooth the wrap with a piece of tissue paper to remove any air bubbles or wrinkles.

6. Place a piece of filter paper on the gel support of the gel dryer to prevent contamination of the dryer by radioactivity.

7. Place the filter paper/gel/plastic wrap sandwich on the filter paper in the gel dryer with the plastic sheet facing up.

8. Position the rubber sealing gasket of the gel dryer over the gel. Set the appropriate heat setting on the gel dryer (normally 80°C; 60°C if the gel contains a fluor). Apply the vacuum and allow the gel to dry (typically 2 hr for a gel of 1 mm thickness).

Removing the gel before it is completely dry can lead to cracking; it is therefore not a good idea to rush the drying process. A rough indication of whether the gel is dry can be obtained by feeling the gel under the sealing gasket. If the gel is dry, it should be warm over the entire surface.

9. Remove gel from dryer and proceed with autoradiography (see Basic Protocol).

USE OF INTENSIFYING SCREENS

SUPPORT PROTOCOL 2

Intensifying screens are used to enhance the film image generated by radioactive molecules (Laskey and Mills, 1977; Laskey, 1980). They are used strictly in conjunction with strong β-emitting isotopes such as ^{32}P or γ-emitting isotopes such as ^{125}I. Emissions from these forms of radiation will frequently pass completely through a film, but they can be absorbed by an intensifying screen which fluoresces and exposes the film with multiple photons of light. While an intensifying screen will substantially enhance the film image as compared with direct exposure (Table 6.3.1), some loss of image resolution will occur due to light scatter. Intensifying screens are distributed by most laboratory supply companies (e.g., Fisher, Sigma, and Kodak).

As shown in Figure 6.3.1, the film should be placed between the sample and the intensifying screen. Preflashed film (see Support Protocol 3) should be used if the sample is weakly radioactive or if quantitation of the radioactivity is desired. The preflashed side of the film should be placed adjacent to the intensifying screen. For very weakly radioactive samples, a second screen can be placed on the other side of the radioactive sample (i.e., screen, then sample, then film, then screen), but this causes further loss in resolution due to light scatter. Also, the sample and sample support must be sufficiently transparent to allow light from the second screen to reach the film. The film should be exposed at −70°C to stabilize the silver bromide crystals activated by the radioactivity or the light emitted from the screen.

Table 6.3.1 Different Methods for Isotope Detection and Their Sensitivities[a]

Isotope	Method[b]	Sensitivity[c]	Enhancement over direct autoradiography[d]
^{125}I	S	100	16
^{32}P	S	50	10.5
^{14}C	F	400	15
^{35}S	F	400	15
^{3}H	F	8000	>100

[a]Exposures conducted at −70°C using preexposed film.

[b]S, intensifying screen; F, fluorography using PPO.

[c]Defined as dpm/cm^2 required for detectable image (A_{540} = 0.02) in 24 hr.

[d]Direct autoradiography for comparison was performed on Kodirex film (Laskey, 1980).

PREFLASHING (PREEXPOSING) FILM

Silver bromide crystals that are activated by light, β particles, or γ rays are highly unstable and quickly revert back to their stable form. The absorption of several photons increases their stability but does not ensure development; approximately five photons of light are required to obtain a 50% probability that any single silver bromide crystal will be developed during film processing. This inefficiency means that film images produced by very low levels of exposure will be disproportionately faint. However, two measures can be taken to maximize efficiency and linearity of exposure at the low levels commonly encountered in ordinary use. First, the film should be preexposed to a hypersensitizing flash of light, which provides several photons per silver bromide crystal and stably activates them without providing enough exposure to cause them to become developed. This allows a linear relationship to be drawn between the film image and the amount of radioactivity in the sample. Second, film exposure should be conducted at low temperatures (−70°C) to slow the reversal of activated silver bromide crystals to their stable form (Laskey and Mills, 1975).

Film can be hypersensitized by exposure to a flash of light (<1 msec) provided by a photographic flash unit or a stroboscope before being placed onto the radioactive sample for exposure of the autoradiogram (Laskey and Mills, 1975, 1977). As the optimal light intensity required for preexposure varies with the type of film and the flash unit being used, the ideal exposure is best determined empirically as described below.

Materials

Stroboscope or flash unit (e.g., Auto 22 Electronic Flash from Vivitar or Sensitize Pre-Flash from Amersham Pharmacia Biotech)
Neutral-density filter (Kodak)
Orange filter (Wratten 22; Kodak)
X-ray film
Spectrophotometer

1. Cover the stroboscope or flash unit with the neutral-density and orange filter.

   This serves to decrease the intensity of emitted light, particularly the blue wavelengths to which X-ray films are most sensitive. Filters are not required for the Amersham flash unit.

2. Place the film perpendicular to the light source at a distance of ≥50 cm to ensure uniform illumination.

3. Expose a series of test films for different flash lengths, then develop them (see Basic Protocol).

   An uneven fog level on film can be remedied by placing a porous paper diffuser, such as Whatman no. 1 filter paper, between the film and the light source.

4. Cut the films into pieces that fit into a cuvette holder of a spectrophotometer and measure the absorbance at 540 nm.

   Choose an exposure time that causes the absorbance of the preexposed film to increase by 0.15 with respect to film that was not preexposed.

FLUOROGRAPHY

ALTERNATE PROTOCOL 1

Organic scintillants can be included in radioactive samples to obtain autoradiograms of weak β-emitting isotopes such as ^{3}H, ^{14}C, and ^{35}S. The scintillant fluoresces upon absorption of β particles from these isotopes, facilitating film exposure. Fluorographs of radioactive molecules in polyacrylamide gels have traditionally used the scintillant PPO (2,5-diphenyloxazole; Laskey and Mills, 1975). PPO, however, has largely been replaced with commercial scintillation formulations that reduce the amount of preparation time and are considerably safer to use. These scintillants (e.g., Enhance from NEN Life Science) come with complete instructions for their use. In addition, spray applicators are also available that can be used on filters or thin-layer plates. The expected levels of image enhancement obtained through fluorography are listed in Table 6.3.1. Sodium salicylate can also be used for fluorography as described below (Chamberlain, 1979). It yields levels of image enhancement comparable to organic scintillants, although it sometimes causes a more diffuse film image. The conditions should work for most standard sizes and thicknesses of gels.

CAUTION: Gloves should be worn at all times; sodium salicylate can elicit allergic reactions and is readily absorbed through the skin.

Materials

Polyacrylamide gel
1 M sodium salicylate, pH 5 to 7, freshly prepared

Additional reagents and equipment for fixing and drying gels (see Support Protocol 1)

1. If gel is acid-fixed, soak for 1 to 5 hr in ~20 vol water to prevent precipitation of salicylic acid from the sodium salicylate.
2. Soak gel 30 min in 10 vol of 1 M sodium salicylate, pH 5 to 7.

   To prevent cracking of gels with >15% acrylamide or thicker than 1.5 mm, 2% (v/v) glycerol can be added to the 1 M sodium salicylate.
3. Dry the gel (see Support Protocol 1) and proceed with autoradiography (see Basic Protocol).

DENSITOMETRY

SUPPORT PROTOCOL 4

Film images obtained by autoradiographic methods can be quantified by densitometry. Densitometers work by comparing the intensity of light transmitted through a sample with the intensity of the incident light. The amount of light transmitted will be proportional to the amount of radioactivity in the gel, provided that the film has been properly preexposed (see Support Protocol 3). The linear range of correctly preexposed film is 0.1 to 1.0 absorbance units. However, if the preexposure is excessive—i.e., an increase of >0.2 absorbance units (A_{540}) treated film/untreated film—smaller amounts of radioactivity will produce disproportionately dense images. Autoradiograms that exceed an absorbance of 1.4 absorbance units (A_{540}) have saturated all available silver bromide crystals and also cannot be evaluated quantitatively.

Densitometers are available from several manufacturers (e.g., Molecular Dynamics, Bio-Rad, and UVP). Most models come with software that facilitates calculations and allows the user to define the region of the film to be measured. Procedures for the use of these machines vary and instructions are provided by the manufacturer. Densitometers are also available that measure light reflected from a sample. Reflectance densitometers are useful in instances where the sample medium is completely opaque—e.g., filters that have been probed using nonradioactive colorimetric detection assays.

ALTERNATE PROTOCOL 2

PHOSPHOR IMAGING

Phosphor imaging screens can be used as an alternative to film for recording and quantifying autoradiographic images (Johnston et al., 1990). They can detect radioisotopes such as ^{32}P, ^{125}I, ^{14}C, ^{35}S, and ^{3}H. There are several advantages of phosphor imaging over film: (1) linear dynamic ranges are 5 orders of magnitude, compared to ~1.5 orders of magnitude for film (Fig. 6.3.2); (2) exposure times are 10 to 250 times faster than with film; (3) quantification is much easier and faster, and most imagers come with software to directly analyze data; (4) fluorography and gel drying are often unnecessary because of the sensitivity of phosphor imaging; and (5) phosphor screens can be reused indefinitely if handled carefully.

Phosphor imaging screens are composed of crystals of $BaFBr:Eu^{+2}$. When the screen is exposed to ionizing radiation such as α, β, or γ radiation, or wavelengths of light shorter than 380 nm, the electrons from Eu^{+2} are excited and then trapped in an "F-center" of the $BaFBr^{-}$ complex; this results in the oxidation of Eu^{+2} to Eu^{+3}, which forms the latent image on the screen. After exposure, the latent image is released by scanning the screen with a laser (633 nm). During scanning, Eu^{3+} reverts back to Eu^{+2}, releasing a photon at 390 nm. The luminescence can then be collected and measured in relation to the position of the scanning laser beam. The result is a representation of the latent image on the storage phosphor imaging plates. The image can then be viewed on a video monitor and analyzed with the aid of appropriate software.

Some companies (e.g., Bio-Rad) offer different screens for use with different isotopes. They vary principally in the protective coating on the screen, which is optimized for low- or high-energy β particles or γ rays. No coating is typically used for weak β emitters such as tritium. More recently, screens have also been developed that measure chemiluminescence. Such screens are particularly valuable for use with many nonradioactive labeling protocols.

The protocol below is for the PhosphorImager system from Molecular Dynamics; other phosphor imaging systems are available from Bio-Rad, Imaging Research, and National Diagnostics.

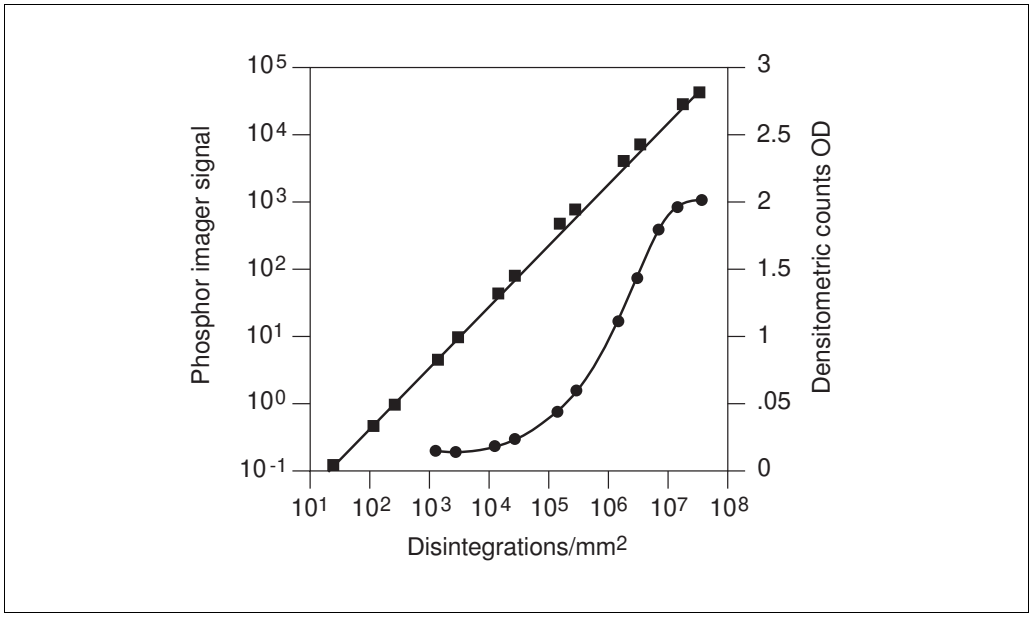

Figure 6.3.2 ^{32}P dilution series quantified on Model GS-525 phosphor imager (squares), compared to film (circles). Image courtesy of Bio-Rad, Hercules, Calif.

Materials

Gel or filter (e.g., from immunoblotting; *UNIT 6.2*)
PhosphorImager system (Molecular Dynamics) including:
ImageEraser light box
Exposure cassette with phosphor screen
Scanning software

1. Erase any latent image on the phosphor screen left by a previous user, or caused by background radiation, by exposure to visible light.

   The PhosphorImager system comes with an extra-bright light box (ImageEraser) for this purpose. Standard laboratory light boxes may also be used.

2. Cover gel or filter with plastic wrap to protect the exposure cassette. Place wrapped gel or filter in the PhosphorImager cassette and close to begin exposure.

   The gel does not have to be dried for this procedure. The phosphor screen is affixed to the lid of the cassette. Exposure times are typically one-tenth of the time required for film exposure.

3. After exposure, slide the screen face down into the PhosphorImager system.

4. Select the scanning area using the software supplied with the PhosphorImager and start scanning.

   The blue light emitted during scanning is collected to produce the latent image.

5. Analyze and quantitate the image using the software provided.

6. Erase the phosphor screen by exposing it to visible light as in step 1.

COMMENTARY

Background Information

The ability to detect radiolabeled proteins is critical to many studies in cell biology. A variety of labeling methods are described throughout this manual, many of which are used to follow protein purification, protein processing, or the movement of proteins within the cell. More often than not, detection of radiolabeled proteins is coupled with the resolving power of SDS polyacrylamide gel electrophoresis (SDS-PAGE; *UNIT 6.1*). Radiolabeled proteins separated on gels can be used directly to obtain an autoradiographic image. Alternatively, proteins separated by SDS-PAGE are frequently transferred to membranes (*UNIT 6.2*) and detected using radiolabeled probes such as antibodies and ^{125}I-labeled protein A. The autoradiographic image, whether generated on film or a phosphor screen, reflects the distribution of the radioactive proteins on the two-dimensional surface of the gel or filter. Molecular sizes of radiolabeled proteins, therefore, can be determined by correlating their positions with molecular markers. Also, the density of the band images can be used to determine the relative quantities of the radiolabeled proteins in the sample.

Critical Parameters

The sensitivity of the detection device and the strength of the radioactive signal are the two most important parameters for autoradiography. Sensitivity can be enhanced by treating samples with fluors or by using intensifying screens (Table 6.3.2). Because phosphor imaging is 10 to 250 times more sensitive than film (Johnston et al., 1990), this technology makes it possible to monitor radioactive samples that would previously have gone undetected with film.

A second important parameter is the range over which the measurement device is linear. Film requires preflashing in order for the intensity of the image to be linear with respect to the amount of radioactivity, particularly for weakly radioactive samples (Laskey and Mills, 1975, 1977). Phosphor imaging offers a much wider linear range of measurement (5 orders of magnitude compared to 1.5 for film; Johnston et al., 1990). This makes it possible to accurately

Table 6.3.2 Film Choice and Exposure Temperature for Autoradiography

Isotope	Enhancement method	Film	Exposure temperature
^{3}H	Fluorography	Double-coated	−70°C
^{35}S, ^{14}C, ^{32}P	None	Single-coated	Room temperature
^{35}S, ^{14}C, ^{32}P	Fluorography	Double-coated	−70°C
^{32}P, ^{125}I	$CaWO_4$ intensifying screens	Double-coated	−70°C

quantitate very weak or very strong radioactive samples.

Troubleshooting

Cracking is one of the most common problems encountered when drying gels. This often occurs if the gel is removed from the dryer before it has adequately dried or if drying temperatures are too high. To overcome this problem, drying times should be extended and the performance of the vacuum pump and heater unit should be checked. For many gels, particularly for those with high percentages of polyacrylamide or >1.5 mm thick, cracking can be reduced by using an alternative fixing solution containing glycerol (3% glycerol/10% glacial acetic acid/20% methanol; see Support Protocol 1).

Among the biggest problems encountered in autoradiography are images that are either too weak or too intense. Such problems can be solved by varying the exposure time. Estimating initial exposure time is difficult, since the amount of radioactivity in the sample is often unknown. A Geiger counter can offer some guidance with certain isotopes. For highly exposed film, the length of time in developer can be reduced to produce a lighter image. It is particularly important to remember that if accurate quantification of the film image is desired, film must be preflashed so that there is a linear relationship between the amount of radioactivity in the sample and the image intensity.

Artifacts, such as black spots and stripes, can be avoided during developing by making sure that no moisture comes in contact with the film and that films exposed at −70°C are brought to room temperature before developing. Also, it must be noted that β particles from weak isotopes such as ^{3}H cannot penetrate plastic wrap, and plastic wraps can attenuate signals from ^{35}S and ^{14}C up to two-fold.

Anticipated Results

The protocols described here should yield a film image of a gel that can be quantified, stored, and photographed for publication.

Time Considerations

Fixing a gel will require ~45 min. Drying will take an additional 2 hr for a gel 1 mm in thickness. Incorporation of a fluor will add ~45 min to the processing time.

For gels >1.5 mm thick or with >15% acrylamide, an additional 30 min will be required for fixing and ~30 additional minutes will be required for drying.

The length of exposure for films in autoradiography can range from a few minutes to a few weeks, depending on the strength of the radioactivity in the sample. Most exposures last from several hours to a few days. Exposure time can be reduced more than 10-fold with a phosphor imager.

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Two-Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis combines two different electrophoretic separating techniques in perpendicular directions to provide a much greater separation of complex protein mixtures than either of the individual procedures. The most common two-dimensional technique uses isoelectric focusing (IEF) (see Basic Protocols 1 and 2) followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a perpendicular direction (see Basic Protocol 3). This combination of isoelectric point (pI) and size separation is the most powerful tool for protein separations currently available. After staining, proteins appear on the final two-dimensional gel as round or elliptical spots instead of the rectangular bands observed on one-dimensional gels. Although the total separating power of large-format two-dimensional gels is estimated to be >5000 spots per gel, in practice a single two-dimensional separation of a complex mixture such as a whole-cell or tissue extract may produce 1000 to 2000 well-resolved spots when a sensitive detection method is used.

Until recently, the most common IEF procedures were based on the use of soluble ampholytes, relatively small organic molecules with various isoelectric points and buffering capacities. The pH gradient for IEF gels is produced when the soluble ampholytes migrate in the gel matrix until they reach their isoelectric point. Because stable pH gradients outside the pH 4.0 to 8.0 range are difficult to create with soluble ampholytes, alternative protocols using nonequilibrium conditions are required to resolve proteins with pI values below 4.0 (see Alternate Protocol 1 for acidic proteins) or above 8.0 (see Alternate Protocol 2 for basic proteins). One of the more important limitations of soluble ampholytes is the difficulty in obtaining highly reproducible pH profiles, especially when very narrow pH ranges are needed.

An increasingly attractive alternative to soluble ampholytes is the use of immobilized pH gradient (IPG) gels (see Basic Protocol 2). In this system, the buffering side chains are covalently incorporated into the acrylamide matrix, and any pH range and curve shape can be generated by pouring a gradient gel using two solutions that differ in ampholyte composition rather than acrylamide concentration. As with tube gels, the initial electrophoresis is followed by a second separation using SDS-PAGE in a perpendicular direction (see Basic Protocol 4). The use of IPG gels has recently increased, for at least three major reasons: many of the technical problems associated with their use have been solved or substantially minimized, reproducible premade IPG gels are now commercially available, and lately strong interest has arisen in using two-dimensional gels for proteome analysis studies (analyzing and comparing the complete protein profiles of cell lines, tissue samples, or single-celled organisms).

Another common two-dimensional electrophoresis format is a nonreducing/reducing electrophoretic separation (see Alternate Protocol 3), which provides useful information about intersubunit disulfides or protein-protein complexes that have been cross-linked using a bifunctional chemical cross-linker containing a disulfide bond within the linker region.

This unit also includes support protocols describing pI standards and pH profile measurements (see Support Protocol 1), electrophoresis of immobilized pH gradient gels (see Support Protocol 2), casting Immobiline gels (see Support Protocol 3), preparation of tissue culture cells and solid tissues for isoelectric focusing (see Support Protocols 4 and 5), preparation of molecular weight standards for two-dimensional gels (see Support Protocol 6), and two-dimensional protein databases (see Support Protocol 7).

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Current Protocols in Cell Biology (1999) 6.4.1-6.4.36

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NOTE: High-purity water (e.g., Milli-Q water or equivalent) is essential for all solutions. For cautions relating to electricity and electrophoresis, see Safety Considerations in the introduction to *UNIT 6.1*.

BASIC PROTOCOL 1

HIGH-RESOLUTION EQUILIBRIUM ISOELECTRIC FOCUSING IN TUBE GELS

This protocol describes the preparation of broad-range first-dimension gels using soluble ampholytes that resolve proteins with pI values between approximately 4.0 and 8.0, and is based on the original procedure described by O'Farrell (1975). The procedure presented here refers specifically to 3-mm IEF tube gels (first-dimension) combined with 1.5-mm-thick 16 × 16–cm (size of separating gel) second-dimension gels (see Basic Protocol 3) and may be easily adapted to a variety of different gel sizes (see Table 6.4.1). A 3-mm IEF gel has a total protein capacity of ~500 µg for complex protein mixtures such as whole-cell extracts. The maximum capacity of any single protein spot is ~0.5 to 5 µg, depending on the solubility of the protein near its isoelectric point and the separation distance from any near neighbors.

In this protocol, gels are cast and prefocused before the sample is loaded. The proteins are then separated according to isoelectric point, and the gels are extruded from the tubes and stored. Measuring pH profiles in IEF gels is a convenient and accurate method for determining pI (see Support Protocol 1). To provide optimal reproducibility, multiple gels should be cast and run simultaneously. This is especially important for comparative studies involving complex mixtures of proteins.

The IEF gels may be cast either by pouring the gel solution into the gel tubes (steps 3a to 7a) or by using hydrostatic pressure (steps 3b to 7b). Pouring the gel solution into the gel tubes is convenient for 3-mm-diameter IEF gels and requires only a minimal excess of reagents. Because the gels are cast using a long needle and syringe, for narrower gels, where the needle does not fit inside the gel tube, casting using hydrostatic pressure is more appropriate. This method requires a larger excess of reagents and special casting cylinders. Many types of ampholytes are readily available from different suppliers to form the desired pH profiles. As ampholytes may vary significantly in their performance, careful selection of the appropriate ampholytes is usually necessary (see Commentary).

Materials

Chromic acid, in acid-resistant container
Urea (ultrapure)
30% acrylamide/0.8% bisacrylamide (see recipe)
20% (w/v) Triton X-100 (see recipe)
Ampholytes (e.g., pH 3-10/2D; ESA)
TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine)
2.5% (w/v) ammonium persulfate (see recipe; prepare immediately before use)
8 M urea (see recipe; prepare immediately before use)
0.1 M orthophosphoric acid (H_3PO_4; see recipe)
0.1 M NaOH (make fresh daily)
Lysis buffer (see recipe)
Protein samples to be analyzed
Equilibration buffer (see recipe)
2-Mercaptoethanol

Isoelectric focusing apparatus (e.g., Protean II xi 2D from Bio-Rad or equivalent) with glass tubes, casting stand, buffer chambers, rubber grommets, and plugs
37°C water bath

continued

110°C oven
10-ml syringe equipped with filter capsule (0.22 or 0.45 µm, e.g., Costar µStar LB)
10-ml syringe equipped with blunt needle [e.g., 20-G × 6 in. (15 cm) or 18-G × 6 in. (15 cm)]
Large glass cylinder sealed at bottom with Parafilm (optional, for hydrostatic pressure casting method only)
2000-V power supply
60-ml syringe
Metal or plastic scoop
Dry ice pellets

Wash tubes and prepare the gel mixture

1. Remove the glass tubes from a chromic acid–filled container. Extensively wash the tubes with water, using high-purity water for the last wash. Dry the tubes at least 1 hr in an oven at 110°C and store them at room temperature, covered with aluminum foil.

   To prevent gels from sticking to the glass tubes, gel tubes have to be very clean. Satisfactory results are obtained by storing the tubes in chromic acid between uses and washing them shortly before use. Because drying the tubes requires at least 1 hr, cleaning steps should be performed the day before gels will be cast.

   CAUTION: *Chromic acid is highly corrosive; follow supplier's precautions carefully.*

2. Prepare the gel solution by mixing:

   16.9 g urea
   4.0 ml of 30% acrylamide/0.8% bisacrylamide
   3.0 ml of 20% (w/v) Triton X-100
   7.5 ml water
   3.0 ml ampholytes.

   Briefly warm the mixture in a 37°C water bath to solubilize urea if needed.

   To minimize decomposition of urea, never warm any solutions containing urea above 37°C, use ultrapure urea, and prepare solutions immediately before use.

   Choice of ampholyte composition is one of the key factors determining the quality of isoelectric focusing separations. Substantial differences in performance, resolution, and shape of the pH gradient formed may be observed with different combinations of ampholytes and with ampholytes from different suppliers. ESA's ampholytes (pH 3-10/2D) are suited for most applications and give reproducible results.

   Although purity of all reagents is important, the purity of urea and choice of ampholytes are among the most critical factors for the quality and performance of isoelectric focusing. Most commercially available reagents marketed specifically for two-dimensional gel electrophoresis should be suitable, although individual lots of reagents from any supplier may provide variability and/or unacceptable results.

Cast gels by pouring

3a. Wrap one end of each glass tube with Parafilm and mount the tube in a casting stand. Mark all the tubes to indicate the desired gel height.

   For reproducible results, all gels should be the same height.

4a. Filter the gel solution using a 10-ml syringe equipped with a syringe-tip filter capsule. Briefly degas the gel solution (~5 min) either by sonication or under vacuum. Then add 42.5 µl TEMED and 187.5 µl of 2.5% (w/v) ammonium persulfate solution to the filtered gel mixture and swirl gently to mix.

5a. Using a 10-ml syringe with a blunt needle, fill each glass tube with gel solution to the desired height. Make sure there are no air bubbles trapped in the gel.

A needle is the best choice for casting gels if tubes of 3-mm inner diameter are used. For narrower tubes, the use of hydrostatic pressure is more appropriate (see steps 3b to 7b, below). For long gels the needle can be extended by inserting a piece of capillary polyethylene tubing over the needle tip. The amount of gel solution described in step 2 is sufficient for sixteen 3-mm tube gels that are 16 cm long.

6a. Immediately overlay each gel with ~50 µl of 8 M urea.

A pipettor with a capillary pipet tip is a convenient tool for overlaying with urea. Avoid mixing the overlay and gel solutions. Polymerization starts to occur ~15 min after the addition of TEMED and ammonium persulfate. It is essential that the gels be poured and overlaid before significant polymerization has occurred.

7a. Let the gels polymerize at least 3 hr prior to use.

Urea decomposes at a substantial rate at room temperature; therefore, the gels should be used the same day they are cast.

Cast gels using hydrostatic pressure

3b. Place a rubber band around the gel tubes so they form a tight bundle. Place the bundle inside a larger glass cylinder that is sealed at the bottom with several layers of Parafilm. All tubes must be precisely vertical.

The dimensions of the larger cylinder depend on the dimensions and number of gel tubes. Excessive space will require more gel solution to cast the gels.

4b. Filter the gel solution using a 10-ml syringe and filter capsule. Degas the gel solution briefly (~5 min) either with sonication or under vacuum. Add 42.5 µl TEMED and 187.5 µl of 2.5% ammonium persulfate solution and swirl.

5b. Pipet the gel solution into the bottom of the glass cylinder. Gently run water down the outside of the tube bundle using a wash bottle. Keep adding water until the gel mix reaches the desired height.

Hydrostatic pressure will force the gel solution into the tubes. Sufficient gel solution must be used to obtain the desired gel height while avoiding forcing any water into the tubes. The volume of gel solution required can be estimated as follows: number of gels × 3.14 × (tube internal radius in cm)^2 × height in cm + ~10 ml to keep a safe level of gel mix at the bottom of the casting cylinder. As water is less dense than the gel solution, the water level will be slightly higher than the level of gel solution inside the tubes.

6b. Overlay the gels with 8 M urea.

Urea decomposes at a substantial rate at room temperature; therefore, the gels should be used the same day they are cast.

7b. Let the gels polymerize at least 3 hr prior to use.

Mount the gels in the electrophoresis unit

8. Prepare the lower electrode solution by degassing the proper amount of 0.1 M H_3PO_4 under vacuum with stirring for at least 5 min. Fill the bottom electrophoresis chamber.

The amount of phosphoric acid depends on the length of the gel tubes and the type of electrophoresis unit. The solution should cover the entire gel for good heat dissipation. Approximately 3 liters are required for Protean II xi 2D electrophoresis units.

9. Remove the gel tubes from the casting stand, remove the Parafilm from the tube bottoms, and inspect gels for irregularities or trapped air bubbles. Discard imperfect gels. If using gels cast with hydrostatic pressure, remove the bundle of tubes en bloc, cut off excess acrylamide with a razor blade, and then rinse away remaining acrylamide particles from the outside of each tube.

10. Place a rubber grommet on the top of the tube. Approximately 5 mm of the tube should be visible above the upper edge of the grommet.

11. Mount the tube with the grommet in the upper reservoir and plug any unused holes.

    After the tube is seated, its lower end must be submerged in the lower electrode solution. Be sure to remove any air bubbles trapped at the bottom of the tube by shaking or tapping the tube gently. Alternatively, with some units bubbles can be dislodged by raising and lowering the tubes or by using a long curved needle and syringe.

Prefocus the gels

12. Prepare the 0.1 M NaOH upper electrode solution by degassing under vacuum with stirring for at least 5 min.

    The amount of upper electrode solution necessary depends on the type of electrophoresis chamber. If a Bio-Rad Protean II xi 2D apparatus is used, 1 liter of 0.1 M NaOH is sufficient for both prefocusing and the separation.

13. Remove the 8 M urea overlay from the top of the gels using a Pasteur pipet and place ~50 µl lysis buffer on the top of each gel.

14. Overlay lysis buffer with the degassed 0.1 M NaOH to fill the gel tubes. Avoid mixing of NaOH with the lysis buffer.

15. Pour the degassed 0.1 M NaOH into the upper chamber, making sure that all the gel tubes are covered with the electrode solution. Check carefully for leaks and air bubbles, then place lid on apparatus.

16. Connect the electrodes to a power supply by the red (+) lead to the lower chamber and the black (–) lead to the upper chamber.

    The voltages and currents used during electrophoresis are dangerous and potentially lethal. Safety considerations are given in the Electricity and Electrophoresis section of UNIT 6.1.

17. Prefocus for 30 min using 500 V constant voltage.

Load the samples

18. Turn off power supply (see Safety Considerations in *UNIT 6.1*), disconnect leads, and remove lid. Using a 60-ml syringe, remove the electrode solution (0.1 M NaOH) from the upper chamber.

19. Remove the electrode solution and the overlay solution from each tube. Be careful not to damage the gel surface.

20. Place ~50 µl lysis buffer on the top of each gel. Wait at least 2 min.

21. Remove the lysis buffer from the tubes.

    Rinsing the gels with lysis buffer removes any residual NaOH and protects the samples against exposure to high pH.

22. Load protein samples to be analyzed and carefully overlay each sample with ~50 µl lysis buffer diluted with water 8:2 (v/v). Avoid mixing the buffer with the sample.

    The overlay solution protects samples from direct contact with the strong base used as an upper electrode solution. Dilution of the lysis buffer with water is necessary to decrease the density so the overlay does not mix with the sample.

    A 3-mm-i.d. × 16-cm-long IEF gel has a total protein capacity of ~500 µg for whole-cell extracts and other complex protein mixtures. The maximum capacity for any single protein spot is ~0.5 to 5 µg, depending on its solubility near its isoelectric point and the separation distance from any near neighbors. Preparation of relatively pure protein samples for

isoelectric focusing is generally straightforward. The sample usually may be prepared in one of the following ways: dialyze into any compatible low-ionic-strength buffer, lyophilize in a volatile or compatible low-ionic-strength buffer and dissolve in lysis buffer, or precipitate the protein using trichloroacetic acid (TCA) and redissolve in lysis buffer. For preparing extracts from cultured cells and from tissue samples, see Support Protocol 4 and Support Protocol 5, respectively.

The minimum sample concentration of protein or radioactivity has to be sufficient for the desired detection method. For complex protein mixtures such as tissue or cell extracts, a 500-µg total load is recommended for Coomassie blue staining or electroblotting (UNIT 6.2) for subsequent structural analysis, a 50-µg total protein load should be sufficient for silver staining or immunoblotting, and no less than 100,000 counts/gel is recommended for proteins labeled with ^{3}H, ^{14}C, or ^{35}S for autoradiography purposes. Sample volumes should be <150 µl for 3-mm gels and <40 µl for 1.5-mm gels. This implies at least a 5 µg/µl protein concentration in the sample for gels to be stained with Coomassie blue.

23. Carefully fill all tubes with 0.1 M NaOH. Avoid mixing the NaOH solution with the overlay solution and the sample.

24. Fill the upper reservoir with 0.1 M NaOH. Be sure that all gel tubes are covered with the solution.

Run the gels

25. Connect the electrodes to a power supply with red (+) to the lower chamber and black (−) to the upper chamber.

26. Focus for a total of 12,000 Vhr.

Unlike other electrophoretic techniques, in IEF the volt-hour is the most common unit describing the "time" of isoelectric focusing. The initial voltage is usually set according to the desired number of volt-hours in a way that is convenient for the operator (i.e., so that the separation will run overnight), but it should not be <400 V. The upper voltage limit is restricted by heat released in the gels during isoelectric focusing. At constant voltage the current will be the highest during the first hour of separation. The initial current will be strongly influenced by the ionic strength of the samples loaded onto the gels. An initial voltage of <800 V is recommended for 3-mm gels loaded with samples containing less than 100 mM salts/buffers; the voltage could be increased to 1200 V after ~1 hr, if cooling is used. The current is a derivative of voltage and is never preset for isoelectric focusing purposes. Some power supplies allow preprogramming the desired number of volt-hours and continuously adjust voltage and current during the isoelectric focusing procedure (constant power). The total number of volt-hours is a major factor that affects separation in the first dimension. Optimal focusing time will vary for different ampholyte combinations, but 12,000 Vhr is a reasonable value for most systems. To achieve a total of 12,000 Vhr set the power supply to 667 V for 18 hr. These conditions are convenient for an overnight separation and do not require use of a cooling unit. Higher voltages can be used but may cause overheating of gels unless a highly efficient cooling system is employed. The maximum practical voltage decreases with increased gel tube inner diameter. Focusing for too long may cause cathodic drift and result in a shifted pH profile in the gel, whereas focusing for a short time will decrease resolution.

Extrude and store gels

27. Turn off power supply and carefully disconnect leads. Detach the lid and remove the NaOH solution from the upper reservoir of the electrophoresis chamber using a 60-ml disposable plastic syringe.

28. Remove one gel tube at a time from the chamber.

29. Using a 10-ml syringe equipped with a blunt needle, slowly and carefully inject water between the gel and glass tube. Start from the bottom of the tube, then repeat the procedure from the top. The gel should slide out of the glass tube.

It is convenient to let the gel slide from the glass tube onto a metal or plastic scoop, which facilitates transfer of the gel into a storage vial. It is relatively easy to break the gel during extrusion, and practicing on several unused gels is recommended. To extrude smaller-diameter gels, use water pressure generated by a syringe connected to the gel tube with Tygon tubing. If clean, unscratched glass tubes are used, extrusion should be easy.

30. Using the scoop, slide the gel into a 4.5-ml cryovial containing 3 ml equilibration buffer and 50 µl 2-mercaptoethanol. Close the vial, incubate exactly 5 min at room temperature, then freeze by placing the tube horizontally on top of dry ice pellets. Do not move or agitate the tube while the sample is freezing.

    The IEF gels may be run on a second-dimension gel immediately (see Basic Protocol 3), or can be stored at −80°C for many weeks. Even when the second-dimension is to be run immediately, extruded gels should be frozen after a carefully controlled incubation time at room temperature, such as the 5 min cited above for 3-mm-i.d. gels, to minimize diffusion of proteins out of the IEF gel. This short incubation before freezing will allow glycerol to diffuse into the gel. Too short an incubation or agitation during freezing can result in gel breakage. The total incubation time in equilibration buffer (sum of the time prior to freezing and after thawing) is critical and should be carefully controlled. Insufficient incubation time in equilibration buffer will not allow sufficient time for SDS to diffuse into the gel and saturate sites on the proteins. Excessive incubation times can result in appreciable protein losses due to diffusion out of the highly porous IEF gel.

CONDUCTING pH PROFILE MEASUREMENTS

SUPPORT PROTOCOL 1

Standards with different isoelectric points can help in evaluating the performance of a specific system and determining the effective pH range in the isoelectric focusing gel. Many pI standards are commercially available from different suppliers. It is most useful to separate a mixture of standard proteins that is prepared from several individual proteins or purchased as a preformulated kit. This mixture should be run in parallel with experimental samples on a separate reference gel. It is generally not recommended to run pI standards together in the same gel with samples because of possible interference with migration and identification of proteins of interest. Instead of analyzing standard proteins, a more precise evaluation of the pH profile can be made by directly measuring the pH throughout the gel using either a surface pH electrode or the following procedure.

1. Prepare and focus one or two gels (see Basic Protocol 1, steps 1 to 26) without any sample in parallel with experimental samples.
2. Prepare 20 to 40 glass test tubes each containing 1 ml high-purity, degassed water for each gel that will be used to measure the pH gradient (measurements on duplicate gels are recommended).

    The number of tubes required per gel equals twice the gel length (in cm).

3. After electric focusing is completed, extrude the blank gels (see Basic Protocol 1, steps 27 to 29). Briefly rinse the gels with water.

    After extrusion, gel surfaces may be contaminated with electrode solutions. Rinsing with water is essential for obtaining reliable pH profiles.

4. Place the gel on a glass plate with a plastic ruler below the plate. Cut the gel into 0.5-cm pieces using a sharp razor blade.
5. Place each gel piece in a test tube containing 1 ml water.

    Do not mix the order of samples because each gel piece represents a single pH profile data point.

6. Place all test tubes on a shaker and shake gently for 1 hr at room temperature.

7. Read the pH of each solution and plot the pH profile as a function of the distance from the top of the gel.

ALTERNATE PROTOCOL 1

NONEQUILIBRIUM ISOELECTRIC FOCUSING OF VERY ACIDIC PROTEINS

Basic Protocol 1 is sufficient for separating proteins with isoelectric points greater than ~3.5 to 4.0. For very acidic proteins, however, a nonequilibrium system is needed. The major features of this method are utilization of a shorter focusing time (without reaching equilibrium), a modified ampholyte mixture, and different electrode solutions.

Additional Materials *(also see Basic Protocol 1)*

10% (w/v) ammonium persulfate (prepare immediately before use)
Concentrated sulfuric acid (used in lower chamber electrode solution)
Ampholytes, pH 2-11 (used in upper chamber electrode solution)

To analyze very acidic proteins, follow Basic Protocol 1 with these exceptions in the indicated steps:

2. When preparing the gel solution, use the following mixture of ampholytes: 2.4 ml ampholytes pH 2.5-4 and 0.6 ml ampholytes pH 2-11.

4. Following the procedure for casting gels by pouring, add 100 µl of 10% ammonium persulfate solution, swirl, add 42.5 µl TEMED, and swirl again.

   Gel mixtures containing entirely or predominantly very acidic or very basic ampholytes are generally difficult to polymerize. Use of an increased ammonium persulfate concentration and adherence to the proper order of adding the reagents should ensure polymerization.

8. Prepare the bottom chamber electrode solution by adding 4.5 ml concentrated sulfuric acid to 3 liters water. Degas at least 5 min.

Omit steps 12 to 19 (do not prefocus the gels).

20. Remove the 8 M urea (polymerization overlay solution) and place ~50 µl lysis buffer on top of each gel. Wait at least 2 min, then remove the lysis buffer.

23. Carefully fill all tubes with the upper chamber electrode (anode) solution prepared by mixing pH 2-11 ampholytes with water in a 1:40 ratio.

24. Fill the upper buffer chamber (anode) with the solution described in step 23.

    Iminodiacetic acid (10 mM) may be a more economical alternative anode solution.

26. Focus for a total of 4000 Vhr.

ALTERNATE PROTOCOL 2

NONEQUILIBRIUM ISOELECTRIC FOCUSING OF BASIC PROTEINS

In general, most equilibrium IEF gel systems using soluble ampholytes produce pH gradients that do not exceed pH 8.0 on the basic end, yet many proteins have higher pI values. For this reason samples containing very basic proteins are usually focused using a nonequilibrium system. In an equilibrium system, proteins are loaded on the basic end of the gel and migrate toward the acidic end until they reach a pH equal to their pI. In nonequilibrium systems, the sample is loaded on the acidic end of the gel, and focusing is terminated after a relatively short time (fewer volt-hours).

To run nonequilibrium IEF gels, follow the procedure previously described (see Basic Protocol 1) with these alterations in the indicated steps:

8. Use 0.1 M NaOH as the lower electrode solution.

   Electrode solutions and electrodes are reversed in this procedure relative to equilibrium isoelectric focusing.

Omit steps 12 to 19 (do not prefocus the gels).

20. Remove the 8 M urea (polymerization overlay solution) and place 50 µl lysis buffer on top of each gel. Wait at least 2 min, then remove the lysis buffer.

23. After loading the samples and overlaying with lysis buffer diluted with water 8:2 (v/v) as in Basic Protocol 1, use 0.1 M H_3PO_4 instead of NaOH to fill all gel tubes.

24. Use 0.1 M H_3PO_4 as the upper electrode solution.

25. Reverse the connection of electrodes—i.e., connect the red (+) lead to the upper chamber and the black (−) lead to the lower chamber.

26. Focus for a total of 3000 to 5000 Vhr.

    The optimal number of volt-hours depends on the nature of the sample and the ampholytes used. The values recommended above may need to be adjusted empirically.

ISOELECTRIC FOCUSING USING IMMOBILIZED pH GRADIENT GEL STRIPS

BASIC PROTOCOL 2

In immobilized pH gradient (IPG) gels, the ampholytes are covalently linked to the acrylamide matrix, which facilitates production of highly reproducible gradients as well as very narrow pH gradients for optimal resolution of minor charge differences. A variety of precast gels and all the necessary equipment are commercially available from either Amersham Pharmacia Biotech or Bio-Rad. Equipment and chemicals are also available for the user to cast gels in the laboratory (see Support Protocol 3), although precast gels are likely to suffice for the majority of applications. Narrow strips of precast IEF gels (Immobiline DryStrips or Ready Strip IPG strips) may be used to achieve a first-dimension separation for two-dimensional gel electrophoresis, and broader precast slab gels (Immobiline DryPlates) can be used to compare multiple samples after IEF separation only (see Support Protocol 2 and Table 6.4.3). In this protocol, precast Immobiline DryStrips from Amersham Pharmacia Biotech are rehydrated overnight using the reswelling cassette (one to twelve sample strips may be handled at a time); samples are applied using sample cup holders and gel strips are isoelectric focused overnight. This procedure has been adapted from instruction booklets provided by Amersham Pharmacia Biotech with Immobiline Dry Strip Kits and with the Immobiline DryStrip reswelling tray. Recently, both Amersham Pharmacia Biotech and Bio-Rad have developed newer IEF systems, the IPGphor Isoelectric Focusing System and the Protean IEF Cell, respectively. These systems simplify IPG strip handling and the overall isoelectric focusing procedure. The IPGphor system also integrates IPG strip reswelling and electrophoresis steps in a single strip holder. These systems include very high-voltage power supplies integrated with efficient cooling units to permit more rapid isoelectric focusing. In general, premade commercial IPG strips from any supplier can be used with any isoelectric focusing device that can physically accommodate the strip. The appropriate user manuals should be consulted for specific details.

Since there is a greater selection of pH ranges for premade Immobiline DryPlates than DryStrips, it is sometimes convenient to cut DryPlates into strips prior to rehydrating the gel to obtain narrower pH ranges where needed. See Basic Protocol 4 for details concerning preparing and running the second-dimension gel.

Wear gloves throughout the procedure and handle the Immobiline DryStrips with forceps where feasible to prevent extraneous protein contamination of the gels and gel solutions.

Table 6.4.1 Rehydration Solutions for Immobiline DryStrips[a]

Component	Final conc.	DryStrip type		
		3-10L	3-10NL	4-7L
Ultrapure urea[b]	7 M	2.1 g	2.1 g	2.1 g
Thiourea[b]	2 M	0.76 g	0.76 g	0.76 g
CHAPS[c]	2%	0.1 g	0.1 g	0.1 g
Pharmalyte pH 3-10	1:50[d]	100 µl		
Pharmalyte pH 4-6.5			50 µl	100 µl
Pharmalyte pH 8-10.5			25 µl	
Ampholine pH 6-8			25 µl	
DTT	0.3%	75 mg	75 mg	75 mg
Bromphenol blue	Trace	A few grains	A few grains	A few grains
Milli-Q water		To 5 ml	To 5 ml	To 5 ml

[a]Rehydration solutions should be prepared fresh immediately before use or stored as frozen aliquots and should be filtered using a 0.2-µm filter. Minimize total time the solution is at room temperature prior to use to minimize decomposition of urea. If the reswelling tray is used, ~250 or 400 µl rehydration solution is required per 11- or 18-cm DryStrip, respectively.

[b]A "total urea" concentration of 9 M is typically used. Thiourea is more effective than urea for minimizing protein precipitation during isoelectric focusing, but its solubility is lower. The combination of 7 M urea/2 M thiourea usually results in superior sample solubilization and isoelectric focusing as compared with 9 M urea alone.

[c]The optimal detergent and detergent concentration should be empirically determined. Other common alternatives are Triton X-100 and octyl-glucoside. The detergent used must be nonionic or zwitterionic to avoid high current and consequent overheating during isoelectrofocusing.

[d]Alternatively, soluble ampholytes are available as preformulated "IPC buffer" concentrates for each type of IPG strip.

Thoroughly clean all equipment with a mild laboratory detergent solution, rinse well with Milli-Q water, and allow to dry before using. Solutions containing 10 M urea may be heated *briefly* to 30° to 40°C to aid in solubilization.

Materials

Urea (ultrapure)
CHAPS or Triton X-100
Pharmalyte 3-10, 4-6.5, and/or 8-10.5 soluble ampholytes (see Table 6.4.1; Amersham Pharmacia Biotech)
Ampholine pH 6-8 (Amersham Pharmacia Biotech)
DTT (dithiothreitol)
Bromphenol blue
Precast Immobiline DryStrips (Amersham Pharmacia Biotech)
DryStrip cover fluid (Amersham Pharmacia Biotech)
Immobiline DryStrip kit (Amersham Pharmacia Biotech) including:
  Cathode electrode
  Anode electrode
  Sample cup bar
  Tray
  Sample cups
  Immobiline strip aligner
  IEF electrode strips
  Sample application pieces
  Instruction manual

continued

Protein sample to be analyzed
Lysis buffer (see recipe)

Immobiline DryStrip reswelling tray (Amersham Pharmacia Biotech)
Forceps
Filter paper
Glass plate
Flatbed electrophoresis unit (Amersham Pharmacia Biotech Multiphor II or equivalent)
Recirculating cooling water bath
Power supply (minimum capacity of 3000 to 3500 V)
Petri dishes

Additional reagents and equipment for protein detection by staining (*APPENDIX 3*) and/or for electroblotting (*UNIT 6.2*, optional)

Rehydrate the Immobiline DryStrip(s)

1. Prepare an appropriate rehydration solution for the type of DryStrips to be used as described in Table 6.4.1 (~400 µl rehydration solution per 18-cm DryStrip).

   The rehydration solution should contain 7 M urea/2 M thiourea, and 2% CHAPS or another appropriate detergent (zwitterionic or nonionic) such as Triton X-100, NP-40, or n-octyl-glucoside should be included in the rehydration solution to aid in sample solubility. The optimal detergent and detergent concentration may vary with type of sample and should be determined empirically.

   One possible method of loading large sample volumes onto IPG gels is to add the sample directly to the rehydration solution. Sample loading during rehydration is preferred when using the IPGphor system with its single strip holder for both rehydration and isoelectric focusing.

   Solutions containing urea should be filtered using a 0.2-µm filter before use.

2. Slide the protective lid off the reswelling tray and level the tray by adjusting the leveling feet until the leveling bubble is centered.

3. For an 18-cm gel, pipet 350 to 400 µl of rehydration solution into a slot of the reswelling tray. Move the pipet along the length of the well while adding the solution to spread it evenly throughout the length of the slot. Avoid excessive air bubble formation while pipetting this solution.

   If the IPGphor system is used, pipet the rehydration solution containing the protein sample into each cleaned ceramic holder.

4. Remove the protective cover from the Immobiline DryStrips and gently place them, gel side down, into the prepared slot.

   To facilitate their removal after rehydration, the strips should be oriented with their pointed ends at the sloped end of the slots in the rehydration tray. Be careful not to trap any air bubbles under the gel strips.

5. Overlay each strip with 2 to 3 ml of DryStrip cover fluid to prevent evaporation and urea crystallization. Slide the protective lid into place and allow gels to rehydrate overnight (~16 hours) at room temperature.

   Shorter rehydration times can be used, although a minimum of 6 to 8 hr is usually needed to completely and reproducibly rehydrate the gels. Do not substantially exceed 16 hr as extensive incubation, especially rehydrating gels over a weekend, increases potential problems due to evaporation and subsequent urea crystallization. In addition, long incubation times increase the extent of urea decomposition, which will increase the risk of amino group modification on proteins by the cyanate produced from urea decomposition.

   If the IPGphor system is used, a low voltage (30 to 40 V) can be applied during rehydration, which improves isoelectric focusing and protein yields of some samples. With this device,

protocols can be preprogrammed so as to allow isoelectric focusing to immediately follow rehydration. Hence, both rehydration with low voltage and isoelectric focusing can be completed overnight, since ~8 hr is sufficient for rehydration, and up to 8000 V can be used in later stages of isoelectric focusing to shorten the total focusing time.

6. After the overnight rehydration, slide the lid off the reswelling tray. Place a forceps tip into the slight depression under each strip and remove the strip. Gently blot any excess oil or moisture from the plastic backing of the rehydrated strips with filter paper. A damp piece of filter paper may also be used to blot the surface of the gel. Some of the paper may adhere to the gel and should be gently peeled away. The gel is now ready to be placed in the strip aligner on the cooling plate of the electrophoresis unit.

   Do not allow the gel to dehydrate prior to placing it on the cooling plate in step 11. (Steps 7 to 10 should be completed prior to removing the strips from the reswelling tray.)

Run the first dimension

7. Level the Multiphor II electrophoresis unit, then connect it to a circulating cooling water bath. Allow it to cool to 15°C for 1 to 2 hr to ensure even cooling. Do not cool below 15°C to prevent precipitation of urea in the gels.
8. Pipet ~5 ml DryStrip cover fluid onto the surface of the Multiphor II cooling plate. Position the Immobiline DryStrip tray on the cooling plate oriented with the red (+, anodic) electrode at the top, near the cooling tubes.

   Avoid large air bubbles between the cooling plate and the tray (small bubbles should not cause a problem).

9. Connect the red and black electrode leads on the tray to their respective positions on the Multiphor II unit. Pour 10 ml of DryStrip cover fluid into the tray. Place the Immobiline DryStrip aligner on top of the oil, groove side up.

   Avoid getting oil on top of the strip aligner. The possible presence of small air bubbles under the strip aligner is not important.

10. Cut two electrode strips to a length of 11 cm (regardless of the number of DryStrips used). Place the electrode strips onto a clean glass plate and soak each one with 0.5 ml Milli-Q water. Blot with a Kimwipe or tissue paper to remove excess water.

    The electrode strips should be evenly soaked and just damp after blotting. Excessive water could cause sample streaking.

11. Transfer the strips from step 6 to adjacent grooves in the aligner tray. Position the rounded (acidic) end of each strip near the top of the tray at the red electrode (anode) near the cooling tubes, and the square end at the bottom of the tray near the black electrode (cathode). Be sure that the edges of all gel strips at the anode end are lined up evenly.
12. Place the blotted electrode strips from step 9 on top of the gel surface of the DryStrips near the anode and cathode ends of the gel. Position the red (anode) and black (cathode) electrodes on top of the electrode strips at their respective ends.

    After the electrodes have been pressed down on top of the electrode strips, check that the gel strips have not shifted position.

13. Push the sample cups onto the sample cup bar. Place the sample cup bar near the anode end of the gel so that the small spacer arm just touches the electrode and the sample cups are nearest to the electrode, but do not allow the cups to touch the gel.

    The sample cups should face the nearest electrode. The acidic end of the gel can usually be used for sample application; however, the optimal loading position may need to be determined empirically for different types of samples. At high protein concentrations and/or at non-optimal pH, samples may precipitate in the gel at the loading position.

14. Position one sample cup above each gel strip and push down to ensure good contact between the bottom of the sample cup and the gel strip. Make sure the gel strips have not shifted position.

15. Pour 70 to 80 ml of DryStrip cover fluid into the tray (it will cover the gels). If oil leaks into the sample cups, adjust the cups to stop leakage. When there is no leakage into the sample cups, add enough cover fluid to the tray to completely cover the sample cups (~150 ml).

16. Pipet protein samples (in lysis buffer) into the sample cups by underlaying. The sample should sink to the bottom of the cup. Check for leakage of the sample out of the sample cup.

    Samples should either be lyophilized and then solubilized in lysis buffer, or diluted 9 parts lysis buffer to 1 part sample. The maximum volume each sample cup holds is 100 µl. The complexity of the sample, the sample solubility at the loading concentration and pH used, the thickness of the second-dimension gel, and the detection method to be employed should be considered when deciding how much protein to load. As a starting reference, typical loading ranges for 1.0- to 1.5-mm-thick 18-cm × 18-cm gels would be ~5 to 20 ng per major spot for silver staining and ~1 to 5 µg per major spot for Coomassie blue staining. When very complex samples are used such as whole cell extracts, total protein loads are likely to be ~20 to 100 µg for silver staining and ~200 to 1000 µg for Coomassie blue staining. The salt concentration in samples should be kept <50 mM and, if the sample contains SDS, the final SDS concentration should be <0.25%.

17. Place the lid on the Multiphor II unit and connect the leads to a power supply. Focus the gels with constant voltage for 2 to 3 hr at 500 V followed by 12 to 16 hr at 3500 V for a total of 40 to 60 kVhr. Refer to the user manual for exact recommended voltage conditions for each type of Immobiline DryStrip.

    The optimal number of Vhr will depend upon the pH range of the Immobiline Strip used, the type of sample, and the sample load and volume; therefore, the optimal Vhr should be empirically determined for different applications.

18. When isoelectric focusing is complete, disconnect the power supply and remove the cover from the Multiphor II unit. Remove the electrodes, electrode strips, and sample cup bar from the tray.

    If gels are to be run in the second dimension immediately after isofocusing, steps 1 to 3 of Basic Protocol 4 should be completed prior to terminating isofocusing.

19. Using forceps, remove the DryStrips from the tray. If the gels are to be run in the second dimension immediately, place in a petri dish with the support film along the wall of the dish and proceed directly to equilibration of the gel (see Basic Protocol 4, step 4). Alternatively, gels may be stored sealed in a plastic bag at −80°C until ready to run the second-dimension gel.

    Gels may be stored at least 2 to 3 months at −80°C. Do not place in the equilibration buffers required for the second dimension prior to storage.

ELECTROPHORESIS ON IMMOBILIZED pH GRADIENT GELS

SUPPORT PROTOCOL 2

In this protocol, after precast IEF gels (Immobiline DryPlates) from Amersham Pharmacia Biotech are rehydrated, samples are loaded and subjected to isoelectric focusing. Gels are typically run at 2500 to 3500 V and require focusing times of 2 to 7 hr. Protein samples may be detected by conventional methods such as Coomassie blue or silver staining. Isoelectric points can be determined with the use of pI calibration proteins; alternatively, because the gradient is linear, one can measure the migration distance across the gel and estimate the pI at each location. As noted in Basic Protocol 2, Immobiline DryPlates can be cut into 3-mm-wide strips to use as the first dimension of two-dimensional gels, since

DryPlates are available in narrower pH ranges than DryStrips. DryPlates can also be used as described in this protocol to simultaneously separate multiple samples in a single dimension. Applications of this method include initial screening of samples to determine the optimal pH gradient prior to running more time-consuming two-dimensional gels, prescreening fractions from a chromatographic purification step prior to running two-dimensional gels, and evaluation of charge heterogeneity of purified proteins.

Additional Materials *(also see Basic Protocol 2)*

Precast DryPlate gel (Amersham Pharmacia Biotech)
Repel-Silane (Amersham Pharmacia Biotech)
Paraffin oil
Protein samples to be analyzed
Reswelling Cassette kit (Amersham Pharmacia Biotech) including:
  125 × 260 × 3–mm glass plate with 0.5-mm U frame
  125 × 260 × 3–mm glass plate
  Silicone tubing
  Pinchcock
  Clamps
  20-ml syringe
Roller (Amersham Pharmacia Biotech)
Whatman no. 1 filter paper

Flatbed electrophoresis unit (Amersham Pharmacia Biotech Multiphor II)
10° *or* 15°C cooling water bath
Electrode strips
Sample applicator strip or sample application pieces
Power supply (minimum capacity 3000 to 3500 V)

Additional reagents and equipment for protein detection by staining (*APPENDIX 3*) and for electroblotting (*UNIT 6.2*; optional)

Rehydrate the gel

1. Remove precast gel from packaging. If the entire gel is not needed, cut off the required number of lanes and reseal the unused gel. Mark the polarity of the gel section to be used by cutting a small triangle off the anode corner. Handle the gel by the support film only.

   It is critical that the lanes are cut from the gel in the proper orientation to preserve the pH gradient (see Fig. 6.4.1), and polarity must be indicated for proper orientation of electrodes later in the procedure.

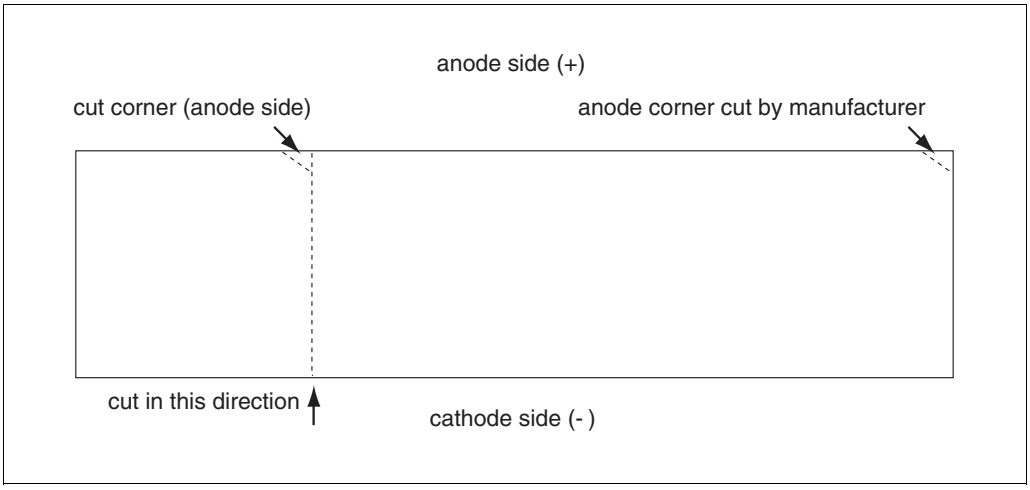

Figure 6.4.1 Marking orientation of a precast IPG gel when only a portion of the gel is used.

2. Use the Reswelling Cassette to rehydrate the gel. Connect silicone tubing through hole in the bottom corner of the U-frame plate, seal with silicone glue, and connect the pinchcock to the other end of the tubing. Place a glass plate on a clean flat surface and wet with a few drops of water. Place the gel on the plate, gel side up. Gently roll with a clean rubber roller to remove any air bubbles.

3. Cover the plate and gel with the plate fitted with the U frame.

   The U-frame plate should be coated with a thin layer of Repel-Silane to prevent the gel from sticking to the plate.

4. Place clamps around the edges of the plates, making sure the seal is tight.

5. Slowly fill the cassette with the desired rehydration solution using a 20-ml syringe connected to the silicone tubing and let stand for the recommended amount of time. Precool electrophoresis unit 1 to 2 hr prior to electrophoresis (see step 9).

   Reswelling with water for 2 to 3 hr is normally sufficient. If using additives such as urea, Triton, glycerol, or reducing agents, allow the gel to rehydrate overnight. Additives can be used to improve solubility of proteins near their isoelectric point. Reducing reagents such as DTT are used to reduce disulfide bonds.

6. When gel has been allowed to rehydrate completely, remove the clamps and gently pry the plates apart.

7. Moisten a piece of filter paper with water and place on top of the gel, then layer with a piece of dry filter paper.

8. Gently blot the gel by rolling over the dry filter paper with the rubber roller to remove excess water. The gel is now ready to be placed on the cooling plate.

   Do not let the gel dehydrate prior to placing it on the cooling plate in step 11.

Run the gel

9. Connect the flatbed electrophoresis unit to a recirculating cooling water bath. Allow to cool to 10°C for 1 to 2 hr to ensure even cooling. If the gel has been rehydrated in the presence of urea, do not cool below 15°C so that urea does not precipitate.

10. Pipet 2 to 3 ml paraffin oil onto the surface of the cooling plate.

11. Position the gel on the cooling plate, being careful not to trap air bubbles between the gel and the plate. Orient the gel so that the polarity of the gel matches the polarity of the cooling plate.

12. Soak two electrode strips with ~3 ml water, then blot to remove excess water.

13. Lay a blotted electrode strip along each long edge of the gel. Cut off the ends of the electrode strip so that it does not extend beyond the edge of the gel.

14. Load protein samples to be analyzed onto the gel. Use an applicator strip for sample volumes between 5 and 20 µl (make sure contact between the strip and the gel is uniform). Use sample application pieces for sample volumes >20 µl. Remove the application pieces halfway through focusing. For sample volumes of 2 to 10 µl, samples may be spotted directly on the gel without using applicator strips. See manufacturer's instructions for further details.

    An important experimental consideration is the position in the pH gradient where the sample is applied. The acidic end of the gel can usually be used for sample application; however, the optimal loading position may need to be determined empirically for different types of samples. At high protein concentrations and/or at nonoptimal pHs, samples may precipitate in the gel at the loading position.

Samples should contain <50 mM salt or buffer components; greater concentrations will cause local overheating of the gel. If possible, salt-free samples should be solubilized or dialyzed in the rehydration buffer.

15. Align the electrodes with the electrode strips, put the safety lid in place, and connect the apparatus to the power supply. Conduct electrophoresis at 3000 V.

    Broad-range IPG gels, such as Immobiline DryPlate, pH 3-10, should be run at ~3000 V for 2 to 4 hr. Narrower-range gels are also run at 3000 V but may require a focusing time of 4 to 7 hr.

16. After removing gels from the electrophoresis apparatus, detect proteins using any conventional staining technique such as Coomassie blue or silver staining.

17. Preserve the gels by sealing in a plastic bag or by drying for a permanent record. Alternatively, electrotransfer the proteins on the gel to a membrane.

    To dry a gel, presoak it first in a preservation solution. For silver-stained gels, use a solution of 5% to 10% (w/v) glycerol/30% (v/v) ethanol; for Coomassie blue–stained gels, use a solution of 5% to 10% (w/v) glycerol/16% (v/v) ethanol/8% (w/v) acetic acid. After soaking the gel, place it on a glass plate gel side up, cover with a cellophane sheet soaked in preservation solution, and allow to dry at room temperature.

    For electrotransfer (UNIT 6.2), use film remover to remove the plastic support film from the gel. Electrotransfer of proteins to a polyvinylidene difluoride (PVDF) membrane using a Multiphor II NovaBlot transfer kit (Amersham Pharmacia Biotech) is recommended. Transferring IPG gels requires special procedures; see the transfer kit manual for instructions.

SUPPORT PROTOCOL 3

CASTING AN IMMOBILINE GEL

An alternative to precast IPG gels is the use of Amersham Pharmacia Biotech Immobilines to cast immobilized pH gradient gels with customized pH gradients and ranges, including very narrow pH ranges, to improve separation of proteins with small charge differences. This protocol describes the general procedure of casting custom-made Immobiline gels. The Reswelling Cassette used in Basic Protocol 2 for rehydrating gels is employed for casting the gels, which are polyacrylamide gels poured with a gradient of Immobilines following instructions provided by Amersham Pharmacia Biotech application note 324.

Additional Materials *(also see Support Protocol 2)*

GelBond PAG film (Amersham Pharmacia Biotech)
Immobiline solutions (Amersham Pharmacia Biotech)
2.5% (v/v) glycerol

Gradient maker
Orbital shaker

Additional materials and equipment for rehydrating immobilized pH gradient gels (see Basic Protocol 2)

Cast the gel

1. Coat the plate with the U frame with Repel-Silane to prevent the gel from sticking to the glass plate.

2. Place a glass plate on a clean, flat surface and wet with several drops of water. Cover with a sheet of GelBond PAG film, hydrophilic side up. Use the roller to remove any air bubbles trapped between the film and the glass plate.

3. Place the plate with the U frame on top of the GelBond PAG film. Clamp the plates together on three sides.

4. Mix the Immobiline solutions following the instructions provided by Amersham Pharmacia Biotech application note 324 to prepare the desired pH range. Cast the pH gradient gel using a gradient maker.

   Once the catalysts have been added to the gel solution, it is important to work quickly to ensure that the gradient is poured before polymerization occurs. See UNIT 6.1 for instructions for using gradient makers.

5. Do not disturb the gel during the first 10 min to allow the gradient to stabilize. Allow the gel to polymerize 1 hr in a 50°C oven.

Dry and store the gel

6. Allow the gel to cool to room temperature, then disassemble the cassette. Cut off a small corner to label the anode end of the gel.

7. Wash the gel 2 to 3 hr with 200 to 300 ml water. Use an orbital shaker and change the water two or three times.

   At this stage the gel may be used immediately (if no additives are needed) or dried as described in steps 8 to 10.

8. Wash the gel 30 min to 1 hr with 200 to 300 ml of 2.5% (v/v) glycerol.

9. Place the gel on a glass plate (gel side up) in a dust-free environment and allow to dry at room temperature overnight.

10. Store the dried gel in a sealed plastic bag at −20°C for up to 2 months.

    The gels may be rehydrated when needed (see Support Protocol 2).

PREPARING TISSUE CULTURE CELL EXTRACTS FOR ISOELECTRIC FOCUSING

SUPPORT PROTOCOL 4

Preparation of samples containing relatively pure proteins for isoelectric focusing is generally straightforward (see Basic Protocol 1, step 22). In contrast, complex samples such as whole-cell extracts, tissue extracts, or subcellular fractions are more difficult to prepare for successful isoelectric focusing. Solubility limitations both prior to isoelectrofocusing and during focusing restrict analysis of these complex samples to protocols that include nonionic detergents and urea (see Basic Protocol 1). In addition, the presence of DNA and RNA in crude cell extracts further complicates isoelectric focusing. The protocol presented below is suitable for preparing samples from cell cultures and is based on quantities compatible with silver staining or Coomassie blue staining. If smaller cell numbers and high-sensitivity detection methods such as autoradiography are used, volumes and quantities should be adjusted as needed.

In this protocol the cells are harvested and washed in phosphate-buffered saline (PBS) with proteolysis inhibitors, then lysed in Tris/SDS buffer using sonication, after which the total protein concentration is determined in the lysate. The lysate is further treated with a mixture of DNase and RNase, and additional SDS and reducing agent are added. At this stage, the samples can be stored at −80°C for an extended time or, after addition of urea and lysis buffer, may be loaded directly onto prefocused IEF gels. Filtration of the final sample prior to loading onto the IEF gel is essential for quality of isoelectric focusing.

Materials

Cell culture flasks containing cells of interest
PBS with proteolysis inhibitors (PBS/I buffer; see recipe)
Dry ice/ethanol (optional, for freezing samples)
Tris/SDS buffer (see recipe)
BCA protein assay kit (Pierce)
DNase and RNase solution (see recipe)
20% (w/v) SDS (*APPENDIX 2E*)
2-Mercaptoethanol
Urea (ultrapure)
Lysis buffer (see recipe)

50-ml centrifuge tube
Centrifuge with rotor (e.g., Beckman JS-4.2), 4°C
1- to 2-ml cryovials
Microcentrifuge, 4°C
Sonicator with microtip
0.2-µm microcentrifuge filter units (e.g., Millipore Ultrafree-MC filter units)

Harvest and wash the cells

1. Place cell culture flasks containing cells of interest on ice.
2. Rapidly wash cells three times with 2 to 6 ml PBS/I buffer. Keep the flasks on ice.

   The required volume of PBS/I buffer depends on the flask size. For example, use 2 ml for a 25-cm^2 tissue culture flask and 6 ml for a 75-cm^2 tissue culture flask.

3. Add 2 to 6 ml PBS/I buffer to the flask, scrape the cells using a cell scraper, and transfer the suspension to a 50-ml centrifuge tube. Repeat this step with another 2 to 6 ml buffer to ensure complete transfer of the cells.

   Cells grown in suspension are washed in an analogous manner using repetitive centrifugation.

4. Collect the cells by centrifuging 15 min at 2600 × *g* (3000 rpm in Beckman JS-4.2 rotor), 4°C.
5. Discard the supernatant and resuspend the cells in a small volume of PBS/I buffer.
6. Transfer the cell suspension to a labeled cryovial.

   The weight of the empty cryovial can be determined prior to use if the wet weight of the cell pellet is desired as a reference value rather than cell number, radioactivity, or another criterion.

7. Microcentrifuge the cells 15 min at maximum speed, 4°C.
8. Remove and discard the supernatant using a pipettor or Pasteur pipet. Weigh the vial containing the cell pellet. Record the wet weight of the cell pellet (in mg).
9. Freeze the cell pellet in a dry ice/ethanol mixture (optional).

   Frozen cells can be stored at −80°C for at least several months.

Prepare the cell pellets for isoelectric focusing

10. Retrieve cell pellets from −80°C storage if samples were frozen.
11. Add 400 µl Tris/SDS buffer per 50 to 100 mg cell pellet wet weight. Keep the cells on ice at all times.

    The total amount of protein in the pellet is roughly 5% of the wet pellet weight.

12. Sonicate the sample three times for 3 sec using a sonicator with a microtip at medium power. Keep the samples on ice during sonication.

    Use pulse sonication or wait at least 5 min between sonications. Minimizing heat generation is essential because substantial proteolysis can occur if the sample warms appreciably. If additional sonication is necessary (i.e., if the sample is not homogeneous), let the sample cool down on ice before the next series of sonications.

13. Run a BCA protein assay to determine the protein concentration if sample will be loaded on that basis. For maximum accuracy use the same amount of Tris/SDS buffer in standards as in experimental samples.

14. Prepare labeled cryovials to store aliquots of the sample, if desired. Precool vials on ice prior to making aliquots.

    The amount of protein per aliquot depends on the anticipated future uses of the sample, as repeated freezing and thawing should be avoided. About 500 µg/gel whole-cell extract is a maximum load for preparative purposes using 3-mm gels (i.e., isolation of proteins for sequencing or other structural work). Approximately 50 µg/gel is an appropriate load for silver staining. The final protein concentration after completion of the protocol (steps 15 to 20) will equal the concentration found by protein assay divided by 1.1, owing to the addition of reagents after the protein assay step.

15. Add 20 µl DNase and RNase solution per 400 µl Tris/SDS buffer used for sonication (step 11). Incubate 10 min on ice.

16. Add 20 µl of 20% SDS solution and 5 µl of 2-mercaptoethanol per 400 µl Tris/SDS buffer used in step 11. Incubate 5 min at 37°C.

17. Quickly divide samples into previously prepared cryovials and immediately freeze aliquots using a dry ice/ethanol bath. Store at −80°C. Samples stored at −80°C are stable ≥1 year.

    Work quickly to minimize potential proteolysis.

    This step may be omitted if the samples are to be loaded on IEF gels immediately. Generally, the total amount of sample greatly exceeds the amount required for an IEF gel, and freezing aliquots is beneficial. To avoid potential reproducibility problems, all samples should be processed identically.

Prepare the samples for isoelectric focusing

18. If cell extracts were frozen, thaw samples and immediately add dry urea to 9 M final concentration.

    The amount of urea (in mg) equals 0.83 times the sample volume (in µl). For example, use 83 mg urea per 100 µl sample. The final volume of the sample (with urea added) equals 1.6 times the initial volume (160 µl in the same example).

19. Add an equal volume of lysis buffer (160 µl in the above example) and warm briefly if necessary to dissolve urea.

20. Filter samples using a 0.2-µm microcentrifuge filter unit by microcentrifuging at maximum speed, room temperature, until the entire sample has passed through the filter. Load the desired volume onto the IEF gel.

    If a 500-µg total protein load per 3-mm gel is desired (a practical maximum load for most whole-cell extracts), the protein concentration determined during the protein assay has to be ≥5 µg/µl. If the sample is less concentrated, the sample volume required will be too large for a 3-mm IEF gel. Alternatively, sample loads can be based on cell numbers, radioactivity, or any other appropriate reference (see Basic Protocol 1, step 22).

SUPPORT PROTOCOL 5

PREPARING PROTEINS IN TISSUE SAMPLES

Tissue samples are usually solubilized in lysis buffer using homogenization. After centrifugation, the protein sample can be loaded onto the first-dimension gel. In general, much higher sample-to-sample variability is expected when tissue samples are analyzed.

Materials

Tissue samples
Lysis buffer (see recipe)

Dounce homogenizer or equivalent
Ultracentrifuge and rotor (e.g., Beckman Ti70), 2°C

1. Place tissue sample in a Dounce homogenizer, add ~2 ml lysis buffer per 100 mg tissue, and homogenize the sample on ice (e.g., 3 to 5 strokes).
2. Let the mixture stand a few minutes, then transfer to an appropriately sized ultracentrifuge tube depending on total sample volume.
3. Centrifuge 2 hr at 100,000 × *g* (e.g., 33,000 rpm in a Beckman Ti70 rotor for 100,000 × *g*), or 1 hr at 200,000 × *g*, 2°C.
4. Divide the supernatant into aliquots and freeze at −80°C or immediately load an appropriate volume onto the IEF gel.

BASIC PROTOCOL 3

SECOND-DIMENSION ELECTROPHORESIS OF IEF TUBE GELS

Second-dimension gels are identical to those described in *UNIT 6.1* except for sample loading, which requires a broad, flat well. A broad well can be cast using an appropriate two-dimensional comb if the second-dimension gel thickness is slightly larger than that of the first-dimension gel. Alternatively, when the second-dimension gel is being cast, water can be layered over the entire surface of the gel to produce a flat surface that will accommodate the first-dimension gel.

Narrow analytical isoelectric focusing gels (≤1.5 mm) that fit between the glass plates of the second-dimension gel do not generally require a stacking gel, although a stacking gel may improve resolution under some circumstances. Stacking gels are essential when first-dimension gels >1.5 mm are loaded on reduced-thickness second-dimension gels, for example, when 3-mm first-dimension gels are loaded on 1.5-mm second-dimension gels. To ensure the best reproducibility, casting multiple second-dimension gels in a multigel casting stand is strongly recommended. This is especially important when gradient gels are used for the second-dimension and/or critical comparisons of multiple samples are planned.

This protocol describes all the specific steps required for successfully casting and running the second-dimension gel. The use of beveled plates and an agarose overlay is especially important when 3-mm IEF gels are loaded onto 1.5-mm second-dimension gels.

Materials

2% (w/v) agarose (see recipe)
Equilibration buffer (see recipe)
Isoelectric focusing gels containing protein samples to be analyzed (see Basic Protocol 1)
Piece of agarose containing molecular weight standards (see Support Protocol 6)

Beveled glass plates
Boiling water bath
Metal or plastic scoop

Additional reagents and equipment for linear and gradient Laemmli gels (*UNIT 6.1*)

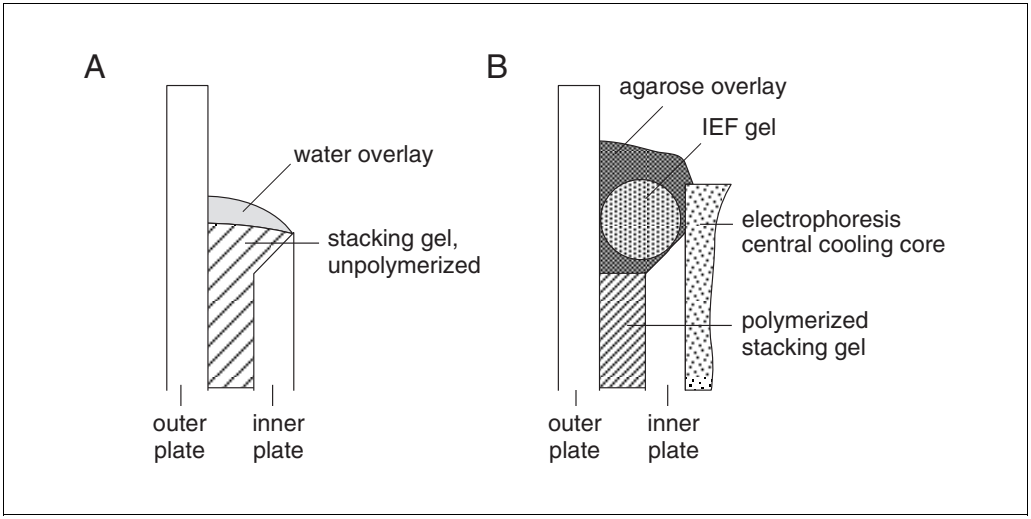

Figure 6.4.2 Casting the second-dimension gel and loading the IEF gel. (**A**) The stacking gel solution should reach to the upper edge of the beveled plate, and then the gel solution has to be overlaid with a minimum volume of water. The water will stay on the surface because of surface tension. (**B**) After polymerization, the gel is mounted on the central cooling core of the electrophoresis unit, and the equilibrated IEF gel is placed on top of the polymerized stacking gel. Excess buffer is removed, and the IEF gel is overlaid with hot agarose/equilibration buffer mixture. After the agarose solidifies, the upper electrophoresis chamber is filled with buffer.

Cast the second-dimension gels

1. Assemble the glass-plate sandwich of an electrophoresis apparatus, using a beveled plate for the shorter side of the gel sandwich.

   A beveled plate provides more space for a thicker IEF gel and will accommodate a first-dimension gel that is at least 1 to 2 mm larger than the thickness of the second-dimension gel.

2. If the thickness of the first-dimension gel exceeds that of the second-dimension gel, pour a separating gel of the desired acrylamide concentration and immediately overlay with water to produce a smooth surface. The separating gel height should be a minimum of 2 cm below the top of the beveled plate to accommodate the stacking gel.

3. After the separating gel has polymerized (a sharp interface between the polymerized gel and the water overlay will reappear), remove the overlay, rinse the gel surface with water, and pour the stacking gel. The stacking gel solution should reach to the top of the bevel. Immediately overlay the stacking gel solution with a minimum amount of water, which will adhere owing to the surface tension (see Fig. 6.4.2A).

   A water overlay of the stacking gel provides a smooth surface and better contact between the IEF gel and second-dimension gel. A small volume of water has to be used to avoid lowering the upper edge of the stacking gel below the edge of the beveled plate. The stacking gel height must be between 1.5 and 2 cm. The solution is filled to the top of the bevel so that after the slight shrinkage that occurs during polymerization the top of the polymerized gel will be near the bottom of the bevel (see Fig. 6.4.2B).

Load the isoelectric focusing gels onto the second-dimension gels

4. Assemble second-dimension gels in an electrophoresis chamber. Do not pour electrophoresis buffer into the upper chamber.

5. Melt 2% (w/v) agarose in a boiling water bath and add an equal volume of equilibration buffer for use in step 11. Keep agarose/equilibration buffer in the boiling water bath until step 11 is completed.

6. Retrieve isoelectric focusing gels containing protein samples to be analyzed from storage. Incubate cryotubes containing frozen IEF gels in a 37°C water bath for 15 min for a 3-mm tube gel. A 5- to 7-min incubation is sufficient for 1.5-mm or thinner IEF gels. Do not agitate during thawing, as vigorous agitation of a partially thawed gel can break the gel.

   During this thawing/equilibration step, SDS in the equilibration solution in which the gels were frozen diffuses into the gel matrix and binds to proteins in the IEF gel. The length of incubation in the equilibration buffer is critical because insufficient saturation of proteins with SDS will contribute to vertical streaks on staining. On the other hand, extended incubation in equilibration buffer will result in excessive loss of proteins owing to diffusion of protein out of the gel, which is especially critical for thin IEF gels. For this reason, it is recommended that after extrusion from the IEF tube IEF gels be initially incubated for 5 min to allow adequate diffusion of glycerol into the gel to minimize gel breakage, followed by freezing on dry ice (see Basic Protocol 1, step 30). This is desirable even if the second-dimension gel will be run directly after isoelectric focusing, as it is the most feasible way of precisely controlling the equilibration time while the remaining gels in the IEF run are extruded.

7. Pour the gel and equilibration solution out of the cryovial onto a metal or plastic scoop. Carefully remove excess equilibration buffer with a pipet.
8. Place a few milliliters of electrophoresis buffer on the top of the second-dimension gel.
9. Slowly slide the IEF gel off the scoop and onto the top of the second-dimension gel. Remove all air bubbles trapped between the gels. Remove excess electrophoresis buffer from the top of the second-dimension gel.

   The basic end of the gel may be placed on either the left or right side of the second-dimension gel. However, once a convention is established, all gels should be oriented the same way. The acidic end of the IEF gel can be recognized in two ways: the bromphenol blue will usually be yellow, and a bulge (increased gel diameter) will be present.

10. Place a piece of agarose containing molecular weight standards (see Support Protocol 6) beside the basic side of the IEF gel (optional).

    Note that when molecular weight standards are used, the isoelectric focusing gel has to be shorter than the width of the second-dimension gel.

11. Carefully overlay the IEF gel (and the gel piece with standard proteins) with the hot agarose/equilibration buffer mixture (~2 ml/gel) prepared in step 5. Let the agarose solidify.

    The agarose prevents the IEF gel from shifting position and ensures good contact between the IEF and second-dimension gels.

12. Carefully pour electrophoresis buffer into the upper reservoir, taking care to avoid disturbing the agarose-covered IEF gel.
13. Connect electrodes and run the gels.

    See UNIT 6.1 for electrophoresis conditions.

BASIC PROTOCOL 4

SECOND-DIMENSION ELECTROPHORESIS OF IPG GELS

In this protocol, vertical gel electrophoresis is used as the second dimension for IPG gels in an analogous manner to the protocol described for the second dimension of IEF tube gels (see Basic Protocol 3). One difference is the use of second-dimension gel spacers or gel apparatus that will accommodate an 18-cm-long Immobiline DryStrip; Bio-Rad offers a conversion kit to increase the gel width from 16 cm to 18 cm, and Amersham Pharmacia Biotech offers the Iso-Dalt gel system. The use of beveled plates is not necessary as the 0.5-mm strips are narrower than the second-dimension gel (1.0 or 1.5 mm thick). Another change involves a two-step equilibration of the strips prior to electrophoresis.

Additional Materials *(also see Basic Protocol 3)*

DryStrip equilibration solutions 1 and 2 (see recipes; prepare fresh in step 4)
Immobiline IPG DryStrip with focused protein (see Basic Protocol 2)
Platform shaker

Cast the second-dimension gel

1. Assemble the glass-plate sandwich of an electrophoresis apparatus, using gel plates wide enough to accommodate an 18-cm-long DryStrip gel.

   Beveled plates are not necessary. If the spacers are not wide enough to accommodate an 18 cm gel, the ends of the gel strip may be trimmed away from the IPG gel so that it will fit on top of the second dimension; however, some very basic or acidic proteins may be lost.

2. Pour a separating gel of the desired acrylamide concentration and immediately overlay with water to produce a smooth surface.

   The separating gel should be a minimum of ~2.5 cm below the top of the inner plate to accommodate a 2-cm stacking gel.

3. After the separating gel has polymerized, remove the water overlay, rinse the gel surface with water to remove any unpolymerized acrylamide, and pour the stacking gel to a height of 0.5 cm from the top of the plate. Overlay with water to produce a smooth surface.

   A water overlay provides a smooth surface for better contact between the Immobiline DryStrip and the second dimension gel. The stacking gel height should be ~2 cm.

Load the Immobiline IPG DryStrip gel onto the second-dimension gel

4. Prepare Immobiline DryStrip equilibration solutions 1 and 2 (see recipe).

5. Assemble the second-dimension gels in a electrophoresis chamber. Do not pour electrophoresis buffer into the upper chamber.

6. Melt 2% (w/v) agarose in a boiling water bath. Mix a solution of 1 part 2% agarose to 2 parts equilibration solution 2.

   Keep agarose/equilibration buffer mixture in boiling water bath until step 11 is completed.

   The agarose prevents the IPG DryStrip from shifting position and ensures good contact between the IEF and second-dimension gels.

7. Using forceps, remove the IPG gels from the electrophoresis tray after isoelectric focusing is complete or from the −80°C freezer (see Basic Protocol 2, step 19) and place each strip in a separate petri dish with the support film side of the strip facing the petri dish wall. Add 15 ml of DryStrip equilibration buffer 1. Cover and place on a platform shaker for 10 min.

   Strips may be run in the second dimension immediately after isofocusing or after storage at −80°C. If the strips have been stored at −80°C, remove them from the freezer, then place in petri dish as stated and continue with the equilibration procedure.

8. Discard equilibration buffer 1 and add 15 ml of equilibration buffer 2. Cover and place on a platform shaker for 10 min.

9. Dampen a piece of filter paper and place on a glass plate. Remove the DryStrips from equilibration buffer 2. Place each strip on its edge on the filter paper to remove any excess buffer.

   Strips should not be left in this position for >10 min, or spot sharpness may be affected.

10. Add a small amount of SDS electrode buffer along the glass plate above the second-dimension gel. Place the DryStrip gel in the well with the gel facing out and the basic side to the left. Push the DryStrip down so that it is firmly in contact with the stacking gel of the second-dimension gel. Remove excess running buffer.
11. Overlay the IPG gel strip with the agarose/equilibration buffer (from step 6) and allow agarose to solidify.
12. Carefully pour electrophoresis buffer into the upper reservoir, taking care to avoid disturbing the agarose-embedded IPG DryStrip.
13. Connect electrodes and run the gels.

    See UNIT 6.1 for electrophoresis conditions.

SUPPORT PROTOCOL 6

PREPARING MOLECULAR WEIGHT STANDARDS FOR TWO-DIMENSIONAL GELS

Molecular weight markers are usually necessary for the identification of proteins or as references to describe experimental proteins on two-dimensional gels. In many cases, molecular weight markers are required only at the beginning of a project. Once the system is established, common proteins in the sample (e.g., actin or tubulin) provide sufficient references for molecular weight identification on subsequent gels. To minimize any differences in migration of the molecular weight standards and isoelectric focused proteins, the standard proteins should be loaded on the second-dimension gel in the same manner as the IEF gel. This protocol describes the preparation of standards in solidified agarose. The agarose pieces may be stored at −80°C for at least a year and provide a convenient source of standards for the second-dimension gel. The procedure described is recommended for 3-mm IEF gels. Narrow standards in solidified agarose (made in tubes ≤1.5 mm in diameter) can be prepared by the same method, but extrusion of the thinner agarose gel without breaking is more difficult. The protocol supplies molecular weight markers containing ~2.5 µg of each standard suitable for Coomassie blue staining or 0.25 µg of each standard for silver staining.

Materials

Molecular weight standards (Table 6.1.2)
1× SDS sample buffer (*UNIT 6.1*)
2% (w/v) agarose (see recipe)

Boiling water bath
Glass tubes (3-mm inner diameter)
Plastic or metal tray

1. Prepare 3 ml molecular weight standards in 1× SDS sample buffer using 250 µg of each standard.

   The stated amount is appropriate for Coomassie blue staining of gels. If silver staining is planned, use 25 µg of each standard.
2. Mix the standards with 2 ml of 2% (w/v) agarose melted in a boiling water bath.
3. Prepare clean glass tubes by wrapping one end with Parafilm. Pour the hot mixture into the tubes and let the agarose solidify.
4. Carefully extrude the agarose from the tubes.
5. Cut agarose rods into 5-mm pieces using a razor blade.
6. Freeze all pieces separately on a plastic or metal tray using dry ice.
7. Collect frozen pieces in a plastic bottle and store at −80°C. The standards may be stored ≥1 year.

DIAGONAL GEL ELECTROPHORESIS (NONREDUCING/ REDUCING GELS)

ALTERNATE PROTOCOL 3

Protein subunit compositions and cross-linked protein complexes can be analyzed by two-dimensional gel electrophoresis using separation under nonreducing conditions in the first dimension followed by reduction of disulfide bonds and separation under reducing conditions in the second dimension. Most proteins will migrate equal distances in both dimensions, forming a diagonal pattern. Proteins containing interchain disulfide bonds will be dissociated into individual subunits and can be resolved in the second-dimension gel.

The approach is similar to that described for two-dimensional gel electrophoresis (see Basic Protocol 3) except, in this protocol, the first-dimension gels are nonreducing (i.e., 2-mercaptoethanol or dithiothreitol is omitted from sample buffer) SDS-denaturing gels instead of isoelectric focusing gels. Use of 1.2-mm tube gels for the first-dimension separation and 1.5-mm slab gels for the second-dimension run is recommended.

Additional Materials *(also see Basic Protocol 3)*

Separating and stacking gel solutions (see Table 6.1.1)
1× SDS sample buffer *without* reducing agents (*UNIT 6.1*)
Reducing buffer (see recipe)
1.5% (w/v) agarose in reducing buffer (see recipe; optional, for securing first-dimension gel on second-dimension gel)
Two-dimensional comb (optional)

Additional reagents and equipment for casting tube gels (see Basic Protocol 1), SDS-PAGE (*UNIT 6.1*), and protein staining (*APPENDIX 3*)

Pour and run the first-dimension gel

1. Clean and dry 1.2-mm glass gel tubes for the first-dimension gel (see Basic Protocol 1, step 1).

2. Prepare a separating gel solution with the desired percentage acrylamide (Table 6.1.1); omit the stacking gel for the first dimension.

   Stacking gels can usually be avoided in the first dimension by keeping sample volumes small (i.e., ≤10 µl).

   Less than 200 µl of gel solution is required to cast a single 1.2-mm tube gel 12 cm in length. Adjust the amounts from Table 6.1.1 accordingly.

3. Cast the first-dimension polyacrylamide gels in 1.2-mm tubes using a syringe with a long needle (see Basic Protocol 1, step 5a). Overlay with water and allow the gels to polymerize.

4. Prepare samples in 1× SDS sample buffer without any reducing reagents (i.e., no 2-mercaptoethanol or DTT). Load the samples and electrophorese until the tracking dye is ~1 cm from the bottom of the tube.

Reduce sample and run the second-dimension gel

5. Extrude the gel from the tube (see Basic Protocol 1, steps 27 to 29).

6. Place the extruded gel in a test tube containing 5 ml reducing buffer. Equilibrate 15 min at 37°C with gentle agitation.

7. Cast the second-dimension separating and stacking gels (see Basic Protocol 3, steps 1 to 3), making sure that the top of the stacking gel is at least 5 mm below the top of

the short glass plate. Layer water across the entire stacking gel or use a two-dimensional comb.

Most two-dimensional gel combs have a separate small well for a standard or reference sample.

The use of beveled plates (see Basic Protocol 3, steps 1 to 3) is not essential but is still preferred because it will facilitate loading of the first-dimension gel. In this procedure, the first-dimension gel will fit between the glass plates if 1.2-mm tubes are used for the first dimension and 1.5-mm gels are used for the second dimension.

8. Load the first-dimension gel onto the second-dimension gel. Remove any air bubbles trapped between the gels.

   If the first-dimension gel does not remain securely in place, it can be embedded using 1.5% (w/v) agarose in reducing buffer.

9. Carefully pour electrophoresis buffer into the upper electrophoresis chamber and electrophorese using voltages and times appropriate for the gel type selected.

   Parameters for electrophoresis are given in UNIT 6.1.

SUPPORT PROTOCOL 7

USING TWO-DIMENSIONAL PROTEIN DATABASES

Computerized image acquisition and manipulation constitute the only practical method for systematic qualitative and quantitative evaluation of complex protein patterns from different samples that are to be compared by high-resolution two-dimensional gel analysis. Examples of experimental applications include comparisons of tumor cells or tissues with appropriate normal controls and comparisons of a single cell line under different experimental conditions.

There are currently a number of commercially available image acquisition/computer systems specifically designed for comparing two-dimensional gels and storing associated information in a database. The systems include both hardware and the necessary software for comparing different gels and producing databases containing the two-dimensional protein patterns, with options for annotating specific spots and producing quantitative comparisons among large numbers of different samples. With most systems, images can be acquired from either stained gels or autoradiographs. The equipment used to obtain two-dimensional gel images includes laser scanners, video cameras, and phosphoimagers. After image acquisition, software running on a microcomputer or workstation is used to refine the image, detect spots, and match spots between different gels.

It is essential that very high-quality, reproducible gels be used for computerized comparisons. The greatest dynamic range in protein abundance for a single two-dimensional gel can be obtained using autoradiography or phosphoimaging (*UNIT 6.3*). With these methods, up to several thousand spots can be compared and tracked. A representative reference gel or a composite image can be stored and used as a reference for future experiments.

Information related to each spot on the two-dimensional pattern, including the quantity of protein in the indicated spot on different gels used in the comparison, can be archived and updated. Other known information related to a specific spot can also be added to the investigator-built database, including the pI, molecular weight, amino acid composition, sequence, and/or identity of the protein and any other important attributes correlated with the indicated spot. A number of research groups, including those of Garrels and Celis (Garrels, 1989; Garrels and Franza, 1989; Celis et al., 1991), have extensively characterized hundreds of spots from specific cell lines and have used multiple methods to characterize proteins of interest. The most definitive methods for establishing the identi-

ties for proteins of interest detected by computer-assisted comparisons are protein sequence analysis and, more recently, mass spectrometry of tryptic fragments. Both methods are compatible with the quantities of protein that can be recovered from two-dimensional gels.

REAGENTS AND SOLUTIONS

*Use Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

30% acrylamide/0.8% bisacrylamide

30 g acrylamide
0.8 g bisacrylamide
H_2O to 100 ml

Filter solution through 0.2- to 0.45-µm filter (e.g., Micro Filtration Systems, cellulose nitrate, 0.2 µm). Store at 4°C (stable at least 3 months).

CAUTION: *Acrylamide is a neurotoxin. Wear gloves and a dust mask when handling solid acrylamide. Wear gloves when working with acrylamide solution. Never pipet acrylamide solutions by mouth.*

Agarose in reducing buffer, 1.5% (w/v)

Mix 0.15 g agarose and 10 ml reducing buffer (see recipe). Heat in boiling water bath until dissolved. Prepare immediately before use.

Agarose, 2% (w/v)

Mix 2 g agarose and 100 ml water. Stir on a hot plate until dissolved. Keeping the solution near 100°C, divide by placing 5-ml aliquots in 25-ml glass screw-cap tubes. Let the aliquots solidify. Store at 4°C (stable at least 3 months).

Ammonium persulfate, 2.5% (w/v)

0.25 g ammonium persulfate
H_2O to 10 ml
Prepare immediately before use

DNase and RNase solution

Mix 2.50 ml of 1 M Tris·Cl, pH 7.0 (*APPENDIX 2A*), 250 µl of 1 M $MgCl_2$, (*APPENDIX 2A*), and 2.2 ml water. Add to 5 mg DNase (Worthington) and dissolve DNase. Add 2.5 mg RNase (in solution; Worthington). Mix well, divide into 50- or 100-µl aliquots, and store at −80°C (stable at least 1 year).

The volume of RNase solution needed is variable and depends on the protein concentration, which is reported on the vial label (in mg/ml). The volume of RNase added should be <200 µl; if a larger volume is used, the amount of H_2O should be decreased proportionally.

Final concentrations are 0.5 M Tris·Cl, pH 7.0, 0.1 M $MgCl_2$, 0.1% (w/v) DNase, and 0.05% RNase.

DryStrip equilibration solutions

20 ml 1 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
72 g ultrapure urea
60 ml glycerol
2 g sodium dodecyl sulfate (SDS)
67 ml Milli-Q-purified water
For solution 1: Add 50 mg DTT per 10 ml of equilibration buffer
For solution 2: Add 0.45 g iodoacetamide and a few grains bromphenol blue per 10 ml of equilibration buffer

Make fresh immediately before use

Final concentrations are 50 mM Tris·Cl, pH 6.8; 6 M urea; 30% glycerol; and 1% SDS in a final volume of 200 ml.

EDTA, 2% (w/v)

2 g Na_2EDTA
H_2O to 100 ml
Adjust to pH 7.0 with NaOH
Store at room temperature (stable several months)

Titrate while dissolving. EDTA is difficult to dissolve without addition of NaOH even when the disodium salt is used.

Equilibration buffer

3 g SDS
7.4 ml 2% (w/v) EDTA, pH 7.0 (see recipe)
10 ml glycerol
2 ml 1.0 M Tris·Cl, pH 8.65 (*APPENDIX 2A*)
0.3 ml bromphenol blue (saturated solution in H_2O)
H_2O to 100 ml
Store at room temperature (stable for several weeks)

Final concentrations are 3% (w/v) SDS, 0.4 mM EDTA, 10% (v/v) glycerol, and 20 mM Tris·Cl, pH 8.65.

Leupeptin, 2 mg/ml

20 mg leupeptin
10 ml water
Divide into convenient volumes
Store at −20°C (stable at least 1 year)

Lysis buffer

2.59 g urea (ultrapure)
1.6 ml H_2O
0.25 ml 2-mercaptoethanol
0.3 ml ampholytes
1.0 ml 20% (w/v) Triton X-100 solution (see recipe)
Prepare immediately before use

Use same ampholytes as for the IEF gel formulation. To dissolve urea, warm the mixture in a 30°C water bath if necessary.

Orthophosphoric acid (H_3PO_4), 0.1 M

13.7 ml 85% phosphoric acid
Water to 2 liters
Make fresh daily

Must be degassed prior to use.

PBS, 10×

152 g NaCl
24 g monobasic sodium phosphate, anhydrous
1600 ml H_2O
Adjust pH to ~6.7 with NaOH
Add H_2O to 2 liters
Store at room temperature (stable at least 1 month)

The 1× solution should be pH 7.3 to 7.5. Final concentrations are 130 mM NaCl and 10 mM sodium phosphate.

PBS with proteolysis inhibitors (PBS/I buffer)

20 ml 10× PBS (see recipe)
20 ml 2% (w/v) EDTA, pH 7.0 (see recipe)

continued

200 µl 0.15 M phenylmethylsulfonyl fluoride (PMSF) in 2-propanol
100 µl 2 mg/ml leupeptin (see recipe)
200 µl 1 mg/ml pepstatin (see recipe)
Adjust to pH 7.2 with HCl
Add H_2O to 200 ml
Prepare immediately before use

Diisopropyl fluorophosphate (DFP) is a better serine protease inhibitor than PMSF at lower temperatures (0° to 4°C); however, although both compounds are toxic, exceptional caution must be exercised with DFP owing to its volatility. If DFP is used, work in a chemical fume hood and carefully follow the supplier's precautions. DFP and PMSF have half-lives on the order of hours in aqueous neutral solutions, and the degradation rate increases rapidly as the pH is increased above neutral. Make aqueous solutions immediately before use. Use of 1 M NaOH is convenient to inactivate residual DFP or PMSF.

Final concentrations are 10 mM sodium phosphate, 130 mM NaCl, 0.2% EDTA, 0.15 mM PMSF, 1µg/ml leupeptin, and 1 µg/ml pepstatin.

Pepstatin, 1 mg/ml

10 mg pepstatin
10 ml anhydrous ethanol
Divide into convenient volumes
Store at −20°C (stable at least 1 year)

Thaw aliquots and mix well immediately before use.

Reducing buffer

0.5 g dithiothreitol (DTT)
0.1 g SDS
1.51 g Tris base
Adjust to pH 6.8 with HCl
Add H_2O to 100 ml
Prepare fresh every time

Final concentrations are 0.5% (w/v) DTT, 0.1% (w/v) SDS, and 125 mM Tris·Cl, pH 6.8.

Tris/SDS buffer

0.3 g SDS
0.6 g Tris base
Adjust to pH 8.0 with HCl
Add H_2O to 100 ml
Divide into 5-ml aliquots
Store at −80°C (stable at least 1 year)

Final concentrations are 0.3% (w/v) SDS and 50 mM Tris·Cl, pH 8.0.

Triton X-100 solution, 20% (w/v)

3 g Triton X-100
12 ml H_2O
Warm in 37°C water bath to dissolve Triton X-100
Store at 4°C (stable ~2 weeks)

Urea, 8 M

0.75 g ultrapure urea
1.0 ml H_2O
Prepare immediately before use

Avoid heating above room temperature.

COMMENTARY

Background Information

Two-dimensional gel electrophoresis, using isoelectric focusing followed by SDS-PAGE, is the single most powerful analytical method currently available for separating complex protein mixtures such as whole-cell or tissue extracts. It is therefore a valuable method for following disease-related changes or for detecting changes in protein expression under diverse experimental conditions. To achieve maximum reproducibility between samples to be compared, multiple gels should be cast and run simultaneously (Anderson and Anderson, 1978a,b).

Despite the exceptionally high resolving power of the method, the total number of proteins that can be resolved in a single two-dimensional gel is only ~1000 to 2000, whereas the total number of proteins in a single mammalian cell type is likely to be at least 10 to 20 times higher. Therefore, the old guideline that a single spot on a two-dimensional gel is a single protein needs to be revised as analytical detection methods improve. Conversely, a single protein (single gene product) can produce multiple, usually adjacent spots in the isoelectric focusing dimension owing to variable degrees of chemical or posttranslational modification. Common examples of variable posttranslational modifications that can usually be detected on two-dimensional gels include phosphorylation, glycosylation, and acetylation. Examples of chemical modifications that cause charge heterogeneity include deamidation of side-chain amines (usually asparagines), oxidation of sensitive side chains, and modification of lysines. Potential modification of lysines by urea is particularly important because urea rapidly decomposes to form cyanate, which readily reacts with amino groups, especially above pH 7. Despite potential side reactions, urea is the most useful IEF additive for maintaining solubility of proteins near their isoelectric points.

Two-dimensional PAGE has become a valuable preparative tool for protein isolation in addition to its historical role as an analytical method. The sensitivity of many protein analysis methods has improved to the point where one or several spots from two-dimensional gels are sufficient for protein identification using mass spectrometry methods.

Commercially available equipment for running two-dimensional gels can be divided into four groups based on size: microgels (Amersham Pharmacia Biotech Phast system), minigels (e.g., Bio-Rad or Amersham Pharmacia Biotech), standard or full-sized gels (e.g., Bio-Rad or Amersham Pharmacia Biotech), and large or "giant" gels (ESA Investigator 2D gel system or Amersham Pharmacia Biotech Iso-Dalt gel system). In general, the larger the gel, the better the final resolution, but as gel size increases so do costs, difficulty of gel handling, and time requirements.

Standard-size gels provide adequate resolution for most applications and are relatively easy to handle. A 3-mm soluble ampholyte tube IEF gel or a 0.5 mm × 3 mm × 18-cm Immobiline gel has a total protein capacity of ~500 µg for complex protein mixtures such as whole-cell extracts. The maximum capacity for any single protein spot is ~0.5 to 5 µg, depending on the solubility of the protein near its isoelectric point and the separation distance from any near neighbors. A variety of alternative gel sizes, their limits, and their advantages are summarized in Table 6.4.2. The lower protein limit for any of the systems is determined strictly by the available detection methods.

Proteins can be detected in two-dimensional gels by the same wide range of techniques used for one-dimensional gels. Autoradiography (*UNIT 6.3*), silver staining, and electroblotting to PVDF membranes (*UNIT 6.2*) followed by colloidal gold or colloidal silver staining or immunodetection (*UNIT 6.2*) are among the most sensitive techniques available. If a larger amount of protein is available, Coomassie blue staining of the gel or amido black staining of a PVDF membrane after electrotransfer (*UNIT 6.2*) would be the detection methods of choice.

The major technical limitation in two-dimensional gel electrophoresis is gel-to-gel variation. Even when extreme care is exercised to produce highly reproducible first- and second-dimension gels, some gel-related variability among gels cast at the same time is likely to persist. Another source of variability includes differences in extraction or recovery of proteins during sample solubilization and handling. Maximizing resolution and reproducibility is especially important if computerized comparisons of two-dimensional gels of complex protein mixtures such as cell or tissue extracts are being attempted.

Isoelectric focusing using soluble ampholytes

Soluble ampholytes are mixtures of low-molecular-weight organic compounds with differing side-chain pK_a values that provide buffering capacity. In an IEF gel, the ampholytes migrate to their isoelectric point, where they provide buffer-

Table 6.4.2 Size Options for Two-Dimensional Gel Electrophoresis

Gel type	First-dimension gel		Second-dimension gel[a]		Purpose	Comments[b]
	Diameter *D* (mm)	Length *L* (cm)	Thickness *T* (mm)	Height *H* (cm)		
Microgels/minigels[c]	<1.5	<10	<*D*	<10	Analytical	1-4
	<1.5	<10	>*D*	<10	Analytical	3-6
	>1.5	<10	<*D*[d]	<10	Analytical/preparative	1,2,7,8
Full-size gels[e]	<1.5	12-18	<*D*	12-18	Analytical	1-4
	<1.5	12-18	>*D*	12-18	Analytical	3-6
	>1.5	12-18	<*D*[d]	12-18	Analytical/preparative	1,2,7,8
Giant gels[f]	<1.5	>20	>*D*	>20	Analytical	4-6,9
	>1.5	>20	<*D*[d]	>20	Analytical/preparative	1-3,7

[a]The second-dimension gel width has to be at least equal to the IEF tube gel height.

[b]Key to comments: (1) tube gel cannot be placed directly on top of second-dimension gel, and use of agarose is recommended; (2) use of stacking gel is recommended; (3) extrusion and handling are relatively difficult; (4) total protein load is limited to usually ≤50 µg for whole-cell or tissue extracts; (5) tube gel can be placed directly on top of second-dimension gel, and use of agarose is not necessary; (6) use of a stacking gel is not necessary; (7) total protein load capacity is relatively large; (8) extrusion and handling are relatively simple; (9) extrusion and handling are very difficult.

[c]Minigel systems provide rapid separations with moderate resolution. Microgels (Phastgels) are precast gels that are slightly smaller than most minigels.

[d]Use of second-dimension gels thicker than 1.5 mm is generally not recommended owing to difficulty with either efficient staining or efficient electroblotting.

[e]Full-size gels provide resolution satisfactory for most applications.

[f]Giant gels provide very good resolution. Specialized equipment is required, such as Investigator 2D (ESA), Iso-Dalt (Hoeffer Pharmacia), or homemade giant-size gel systems.

ing capacity and hence produce stable pH gradients. In theory, any desired pH gradient could be produced by blending ampholytes with appropriate pK_a values. In practice, it is relatively easy to produce pH gradients from ~pH 3.5 or 4.0 to pH 8.0, but stable soluble gradients outside this range are usually not technically feasible. Within these pH limits, some manipulation of the gradient shape and pH range is possible by blending different amounts of specific pH range ampholytes. For example, 0.50 ml of pH 5-7 ampholytes plus 0.25 ml of pH 4-8 ampholytes can be used instead of 0.75 ml of pH 4-8 ampholytes alone to increase the separation distance of proteins in the pH 5.0 to 7.0 range.

Basic Protocol 1 is based on use of 3-mm first-dimension isoelectric focusing gels and 1.5-mm second-dimension gels using the Bio-Rad two-dimensional gel apparatus (Protean II xi 2D). The method can be easily adapted to equipment from other suppliers or to different-sized gels by adjusting the quantities of reagents used. The protocol uses 8 M urea and Triton X-100 as solubilizing agents. Solubilization of the protein sample applied to the gel as well as maintenance of solubility during electric focusing are the most critical factors influencing the quality of separation in the first dimension. The most common modification to Triton X-100–based procedures is addition of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) to the gel and solubilizing buffer mixtures. Addition of SDS to complex samples such as tissue or cell extracts can also enhance reproducible solubilization of the largest possible subset of proteins. Although SDS is charged, in the presence of Triton X-100 it is separated from proteins during focusing and migrates to the acidic end of the gel. Regardless of the method used to maintain solubility, some proteins (especially those >100 kDa) tend to precipitate at pH values approaching their isoelectric point and thus produce horizontal smears on the final second-dimension gel.

Basic Protocol 1 requires several modifications for successful separation of very acidic (Alternate Protocol 1) or very basic (Alternate Protocol 2) proteins. In both cases, prefocusing of the gels must be avoided, and the isoelectrofocusing time has to be reduced. A short separation time does not allow the system to reach equilibrium and is used to establish the desired

Table 6.4.3 Commercially Available Precast IPG Gels and Immobiline Chemicals

Name	Use	Available pH range
Immobiline DryPlate[a]	Running one-dimensional immobilized pH gradient gels	pH 4.0-7.0, pH 4.2-4.9, pH 4.5-5.4, pH 5.0-6.0, pH 5.6-6.6
Immobiline DryStrip[a]	Running first dimension in two-dimensional gels	
110 mm		pH 4-7L, pH 3-10L
180 mm		pH 4-7L, pH 3-10L[c], pH 3-10NL[d]
IPG Ready Strips[b]	Running first dimension in two-dimensional gels	
7 cm		pH 3-10, pH 4-7, pH 3-6, pH 5-8, pH 7-10
11 cm		pH 3-10, pH 4-7, pH 3-6, pH 5-8, pH 7-10
Immobiline II[a]	Creating custom gradient immobilized pHgradient gels	pK 3.6, pK 4.6, pK 6.2, pK 7.0, pK 8.5, pK 9.3

[a]From Amersham Pharmacia Biotech.

[b]From Bio-Rad.

[c]A linear gradient with maximum resolution above pH 7.0.

[d]A nonlinear gradient with best resolution at pH 5.0-7.0.

pH gradient. Separation of very acidic proteins requires modifications of gel and electrode solutions. Separation of very basic proteins has to be performed with the positions of electrodes and electrode solutions reversed in the electrophoresis chamber.

The total protein load per gel depends on the complexity of the sample, the solubility of proteins in the sample, and the diameter of the first-dimension gel. Approximately 4 times as much total sample can be applied to a 3-mm gel compared with a 1.5-mm gel. Another advantage of a larger-diameter isoelectric focusing gel is that extrusion and gel handling are easier owing to improved strength of the gel. If care is exercised in loading the first-dimension gel onto the 1.5-mm second-dimension gel, the final resolution will be similar to that obtained using smaller-diameter IEF gels. The separating or resolving power of a system is dependent on the quality of ampholytes used, the slope of the pH gradient, and the lengths of both first- and second-dimension gels.

Immobilized pH gradient gels

In immobilized pH gradient (IPG) gels (Basic Protocol 2), the pH gradient is an integral part of the polyacrylamide matrix (Strahler and Hanash, 1991). Because the pH gradient is covalently associated with the polyacrylamide gel matrix, precise, reproducible, and very high-resolution separations can be achieved. A variety of precast gels and all the necessary equipment are commercially available from either Amersham Pharmacia Biotech or Bio-Rad. Reproducible two-dimensional gels can be obtained by running a sample on a narrow strip of immobilized pH gradient gel and then running it in a vertical SDS second-dimension gel. Isoelectrofocusing is performed in a horizontal electrophoresis unit in which multiple gel strips may be run simultaneously. Equipment and reagents are also available for the user to cast custom Immobiline gels in the laboratory.

Some of the major advantages of using precast IPG gels are their ease of use and high reproducibility, the time savings realized, and the fact that precision narrow-range gradients can be used to resolve small charge differences. Because the pH gradient is covalently coupled to the polyacrylamide gel matrix, the pH gradient remains stable and linear during prolonged electrophoresis, thus ensuring reproducibility. This is in contrast to conventional IEF gels, where gradient drift occurs during prolonged electrophoresis. Additionally, the precast gels can be rehydrated in water or in solutions with one or more additives such as urea, CHAPS or Triton X-100, carrier ampholytes, glycerol, and reducing agents, which may help to increase protein solubility. Precast gel strips are available with pH ranges such as pH 3.0 to 10.0 or pH 4.0 to 7.0 as well as narrower ranges (as DryPlates). In addition, a variety of Immobilines permits the user to cast IPG gels with customized pH gradients of any gradient range and shape between pH 3.0 and pH 10.0.

Table 6.4.3 lists types of commercially available gels and Immobilines. With the Immobiline

system, the apparent pI of a given protein may be slightly different from that determined by other methods. Therefore, it is recommended that a broad-range gradient be tried initially, followed by a narrower-range gradient, if needed.

Diagonal gel electrophoresis

Diagonal gel electrophoresis is a form of two-dimensional analysis useful for investigating the subunit composition of multisubunit proteins containing interchain disulfide bonds (Goverman and Lewis, 1991). Proteins are electrophoresed in the first-dimension in a tube gel (or a slab gel) under nonreducing conditions. The proteins are then reduced in situ, and the first gel (or a strip thereof) is layered onto a second gel and electrophoresed. In the second gel, the proteins migrate at right angles to the original, first-dimension migration. Most cellular proteins are not disulfide-linked and will fall on the "diagonal" in this system; that is, they migrate approximately equal distances in both directions during electrophoresis and lie approximately on the diagonal line connecting opposite corners of the gel. On reduction, component subunits of proteins connected by interchain disulfide bonds will resolve below the diagonal because the individual subunits migrate faster than the disulfide-linked complex during the second electrophoresis. Some proteins with internal disulfide bonds, but no interchain disulfides, may migrate slightly above the diagonal because internal disulfides can produce a more compact molecular shape (causing faster migration in the first dimension).

Investigator 2D gel system

The Investigator 2D gel system was introduced in 1990 by Millipore as the first commercial "large" or "giant" format two-dimensional gel system designed for analytical purposes, although analogous homemade units had been reported earlier (Garrels, 1979; Young et al., 1983). This product line, including precast first- and second-dimension gels, can now be purchased from ESA. The Investigator 2D gel system uses larger gel sizes than the gels described in this unit as well as a number of novel approaches and reagents designed to enhance resolution and gel-to-gel reproducibility. The major features of this system include the following: an increased length of the first-dimension gel (20 cm), an increased length and width of the second-dimension gel (20 $\times$ 22 cm), a thread reinforcement of the isoelectric focusing gel, temperature control during electrophoresis of the second-dimension gel, and use of a special high-tensile-strength acrylamide to facilitate handling of the large, thin second-dimension gel. Narrow gel tubes (1.2 mm) for the first-dimension are standard, but 3-mm-inner-diameter tubes are available to accommodate larger protein loads. The 3-mm IEF gels do not have the thread reinforcement. One modification related to the above protocol includes addition of 0.3% (w/v) CHAPS in the gel solution, which is intended to improve the solubility of proteins and their migration and separation in the gel. The system is supplied with a manual that adequately describes the method.

Critical Parameters and Troubleshooting

Although no individual steps in two-dimensional gel analysis are exceptionally difficult, the large number of steps involved increase the likelihood and possible severity of errors or problems. Several steps are especially critical and may require optimization. The first is sample preparation. Proteins applied to IEF gels have to be completely solubilized. Residual precipitate or even soluble aggregates are likely to cause artifacts on the end of the tube gel or position on the IPG strip where the sample is loaded. Precipitated or aggregated proteins may also interact with soluble proteins, causing components with normally good solubility to coprecipitate or migrate anomalously.

Two general rules of thumb apply to sample solubility: (1) the more complex the sample or the more crude the extract, the more likely problems will be encountered with sample solubility, and (2) the higher the protein load applied to the gel, the more likely solubility problems will arise. Care must also be taken to avoid proteolysis during sample preparation, especially when complex, impure samples are used. If samples are frozen either before or after solubilization, they should be stored at −80°C and should not be subjected to repeated freeze-thawing. Addition of SDS to the solubilization solution increases the solubilization of some proteins; however, SDS should only be used when the IEF gels contain urea and Triton X-100, as the solubilizing agents are required for effective separation of the strongly anionic SDS molecules from the proteins during isoelectric focusing. Heating of samples in urea-containing solutions must be avoided because urea readily decomposes to cyanate, which reacts with amino groups and causes charge heterogeneity. High concentrations of salts and buffers in the sample should be avoided. Ionic compounds increase the conductivity of the sample and can result in localized overheating, especially for Immobiline gels.

Samples analyzed on immobilized pH gradient gels should not contain precipitates. The concentration of salts and buffer ions in the sample should be kept to a minimum (<50 mM) to avoid local overheating of the gel during electrophoresis. If the sample forms aggregates or precipitates at the point of application, apply the sample to a different location (different pH) on the gel, or load the sample during the rehydration step.

Early decisions that must be made include the size of the gels required and whether a soluble ampholyte system or an Immobiline gel will be used. The major consideration affecting appropriate gel size is the degree of resolution needed. In general, the smallest gel format should be selected that will provide the needed degree of resolution, because smaller gels are easier, less expensive, and faster to run. Therefore, quick screening of samples or analysis of relatively simple samples can easily be accomplished with microgels or minigels. In contrast, if detailed qualitative or quantitative comparisons of cell or tissue extracts are planned, standard-size or large gels are indicated. Similarly, 3-mm or larger first-dimension tube gels followed by 1.5-mm second-dimension gels in the standard or large format are indicated if the two-dimensional gels will be used for preparative isolation of a protein for applications such as raising antibodies or conducting structural analysis. Immobilized pH gradient gels should very seriously be considered as an alternative to soluble ampholyte gels for most separations owing to their stable and reproducible pH profiles. The IPG gels are particularly appropriate when a narrow pH range is required.

Immobilized pH gradient gels are typically run at 2500 to 3500 V and typically require a focusing time of 16 to 18 hr. Use of high voltage (8,000 to 10,000 V) integrated power supplies/cooling units can shorten focusing times to <4 hr. Optimal focusing conditions may be experimentally determined by applying the sample to different positions on the gel and estimating the time for the migration patterns to coincide. Some proteins may require longer run times to reach their pI. Because there is no gradient drift, the potential problems with longer run times are limited to sample modifications or drying out of the gel. These problems usually can be minimized by including a reducing agent in the rehydration solution and coating the top surface of the gel with paraffin oil.

The quality of the first-dimensional separation is strongly dependent on the purity of the reagents used, especially the urea and ampholytes. One fairly commonly encountered frustration, when soluble ampholyte gels are used, is that different batches of ampholytes from the same supplier will sometimes produce markedly different two-dimensional gel patterns. Therefore, it is advisable to purchase an adequate supply of a single lot of ampholytes to meet anticipated needs for an entire study where such an approach is feasible. However, whereas ampholytes usually have a reasonably long shelf life at 4°C (usually up to a year), shelf life as well as total ampholyte requirements often cannot be predicted with much certainty.

When any doubt arises about the purity or quality of ampholytes or any other reagent, the reagent should be replaced immediately. Constant monitoring of the system performance, especially when changing lots of ampholytes, urea, or acrylamide, can help minimize potential reagent-associated problems. In most cases, the best standard for a given two-dimensional gel system is an experimental sample or control that is available in sufficient quantity so that many replicate aliquots can be frozen and stored for an extended time at −80°C; alternatively, a sample that can be reproducibly prepared over a long time frame would make an acceptable standard. Such an experimental standard or reference is more likely to detect subtle, but experimentally important, changes in the two-dimensional gel system than commercially available IEF or SDS gel standard mixes.

Another critical factor is equilibration of the first-dimension gel in the second-dimension equilibration buffer. During this step, urea diffuses out of the IEF gel while SDS and reducing reagent diffuse into the gel. If the gel is inadequately saturated with SDS, vertical streaking will result. However, if the gel is incubated in the equilibration buffer for an extended time, a substantial amount of the protein can rapidly diffuse out of the large-pore IEF gel. Losses arising from diffusion can be critical for any experiment because different proteins will diffuse at different rates, but rigorous control of the incubation step is especially important if quantitative comparisons among gels are planned. The simplest method of controlling the incubation time is to freeze the extruded IEF gel after a carefully controlled 5-min incubation in the equilibration buffer; any additional equilibration incubation time required can then be incorporated and carefully controlled when the sample is thawed for loading onto the second-dimension gel.

Another crucial step is loading of the equilibrated IEF gel onto the top of the second-dimension gel. Any irregularity or obstruction between the two gels, including particles of dirt or air bubbles, will affect the flow of current and disrupt

the resolution of proteins in the final gel. Similarly, poor contact or any movement of the IEF gel during electrophoresis of the proteins out of the IEF gel into the second-dimension gel will lead to artifacts. Therefore, it is advisable to embed the IEF gel in a buffered agarose matrix to ensure good electrical contact between the gels and to prevent gel movement after electrophoresis is initiated.

Finally, the choice of second-dimension gel composition and separation conditions can influence the quality of results. A proper percentage of acrylamide should be selected to optimize resolution within the desired molecular mass range. If gradient gels are needed, use of a multiple gel casting stand is the best way to ensure reproducibility among samples within a single experiment. Further details on optimization of two-dimensional gel systems are presented by Hochstrasser et al. (1988).

When no technical, sample-related, or reagent-related problems are encountered, the final stained two-dimensional gels should contain numerous rounded or slightly elliptical spots. Typically, more than 1000 spots can be detected on a standard 16 × 14–cm gel when using a sensitive staining protocol such as silver staining or autoradiography and a whole-cell extract as a sample. Some horizontal streaks for most high-molecular-mass proteins (proteins exceeding ~100 kDa) are common owing to the decreased solubility of larger proteins near their isoelectric points, even in the presence of urea and nonionic detergent. However, excessive horizontal smearing of proteins smaller than 100 kDa indicates poor isoelectric focusing, which could be related to one or more of the following factors: sample improperly solubilized or contaminated with interfering substances such as large nucleic acid molecules, poor purity of reagents (check the urea first), poor-quality ampholytes, or insufficient isoelectric focusing (total volt-hours too low). It is important to note that in general the solubility of any protein is the lowest near its isoelectric point, but there are vast differences among proteins in terms of both the minimum concentration where precipitation becomes a problem and the degree to which precipitation can be prevented by adding different solubilization agents. The best conditions for maintaining solubility during isoelectric focusing for a given sample type must be determined empirically, although the most universal conditions are those described in the protocols in this unit. In contrast, IEF systems that do not use any detergents or denaturants are limited to that fairly small percentage of proteins which maintain good solubility near their isoelectric point.

If high-molecular-weight proteins are expected but are not present in the final two-dimensional gel, check the sample preparation protocol as well as the sample storage conditions. The most likely problem is proteolysis during sample preparation. Multiple freeze-thawing cycles could contribute to this problem. Vertical smears on the two-dimensional gel suggest (1) insufficient equilibration of the IEF gel (not enough SDS bound to the proteins), (2) poor contact between the IEF and second-dimension gels, or (3) problems related to the stacking gel (too short or wrong buffer). Use of a stacking gel is especially important when large-diameter IEF gels are loaded onto smaller second-dimension gels.

Omission of Triton X-100 or other nonionic or zwitterionic detergent from the final sample loaded on the gel can yield poor results, especially for samples containing SDS, because the amount of detergent in the IEF gels alone may be insufficient to remove bound SDS from proteins. The presence of Triton X-100 in the sample is especially important when SDS sample buffer is used to solubilize protein samples (i.e., after immunoprecipitation). The amount of Triton X-100 in the lysis buffer is normally sufficient for effective dissociation of SDS from proteins. If poor results are encountered with SDS-containing samples, try decreasing the final SDS concentration and/or increasing the final Triton X-100 concentration in the sample.

If no proteins are detected on the gel, check whether (1) the total protein load is appropriate for the detection method used, (2) the orientation of electrical connections is wrong or electrical connection during isoelectric focusing is poor (all gels from that run will be blank), (3) an air bubble obstructs current in a single IEF tube, or (4) the electrical connection is incorrect or is poor during the second-dimension gel separation. Careful monitoring of current and voltage at the beginning, during, and at the end of electrophoretic separations is strongly recommended. Recording the initial and final current and voltage will also facilitate troubleshooting. Additional guidelines for troubleshooting and evaluating artifacts in two-dimensional gel electrophoresis are described by Dunbar (1987).

Anticipated Results

A two-dimensional electrophoretic separation of proteins should produce a pattern of round or elliptical spots separated from one another. The pI range of the separated proteins as well as the observed molecular weight range depend on the first-dimension isoelectric focusing protocol and the percentage of acrylamide used for the second-

dimension gel. A complex protein mixture such as a whole-cell extract should produce more than 1000 silver-stained spots distributed over most of the gel area. Fewer spots will be detected with less sensitive detection techniques such as Coomassie blue staining. On the other hand, separation of radiolabeled proteins and use of multiple exposures permit detection of many low-abundance proteins.

Time Considerations

Time requirements are very dependent on gel size and whether an external cooling unit is used to permit faster separations. Isoelectric focusing using the standard-size gel format described in Basic Protocol 1 with 3-mm tubes is most conveniently done in an overnight run of ~16 to 18 hr. This separation time can be decreased to ~5 to 6 hr using higher voltages and an external cooling device. Isoelectric focusing of 18-cm-long IPG gels requires ~16 to 18 hr. Extruding a set of sixteen IEF tube gels and freezing them in equilibration buffer takes 1 to 2 hr, including setup time. Preparing and running SDS gels is described in UNIT 6.1. It takes ~30 min to thaw, equilibrate, and load two second-dimension gels.

Overall, if standard-size gels are used without external cooling, it will take ~3 working days before the results of two-dimensional electrophoresis are obtained. A single person can conveniently run about 16 two-dimensional gels in one week, depending on the amount of electrophoresis equipment available. The rate-limiting step in most laboratories is running the second-dimension gels because 16 soluble ampholyte IEF gels or 12 IPG gels can be focused in one run, but loading, running, and detecting results from 12 to 16 second-dimension gels requires substantial operator time and electrophoresis equipment.

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Key Reference

Hochstrasser et al., 1988. See above.

Discusses methods for improving and troubleshooting two-dimensional separation.

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One-Dimensional Electrophoresis Using Nondenaturing Conditions

Nondenaturing or "native" electrophoresis—i.e., electrophoresis in the absence of denaturants such as detergents and urea—is an often-overlooked technique for determining the native size, subunit structure, and optimal separation of a protein. Because mobility depends on the size, shape, and intrinsic charge of the protein, nondenaturing electrophoresis provides a set of separation parameters distinctly different from mainly size-dependent denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; *UNITS 6.1*) and charge-dependent isoelectric focusing (IEF; *UNIT 6.4*). Two protocols are presented below. Continuous PAGE (see Basic Protocol) is highly flexible, permitting cationic and anionic electrophoresis over a full range of pH. The discontinuous procedure (see Alternate Protocol) is limited to proteins negatively charged at neutral pH but provides high resolution for accurate size calibration.

CONTINUOUS ELECTROPHORESIS IN NONDENATURING POLYACRYLAMIDE GELS

BASIC PROTOCOL

Separation of proteins by nondenaturing electrophoresis requires the same type of equipment used for denaturing slab gels (*UNIT 6.1*) and is adaptable to a range of gel sizes (e.g., from 7.3 × 8.3–cm minigels to 14 × 16–cm full-size gels) and matrix types (e.g., single-concentration and gradient gels). This protocol outlines straightforward procedures for making acrylamide solutions, casting separating gels (stacking gels are omitted), loading samples, and conducting electrophoresis. Continuous systems, although flexible, do not give the high-resolution separation found in discontinuous systems (see Alternate Protocol).

Separation in a continuous system (i.e., in which the same buffer is used for preparing acrylamide solutions and filling electrophoresis chambers) is governed by pH, and this protocol describes four types of buffers useful over discrete ranges from pH 3.7 to pH 10.6. Use of unadjusted acetic acid gel buffer can extend the range to pH 2.0. The choice of pH and thus the buffer system depends on the protein being studied (i.e., its isoelectric point) and often must be determined empirically. In general, the system should be between pH 5.0 and 8.0 for optimal results. Extremes of pH can lead to precipitation or denaturation of the protein. Acrylamide concentrations are empirically determined, but the higher the percent acrylamide, the sharper the protein bands.

It is important to include native protein standards in the electrophoresis runs. Several manufacturers supply standards for isoelectric focusing that are also suitable for native electrophoresis. The standards have a range of isoelectric points and will carry a net positive, negative, or zero charge depending on the pH of the gel system. Alternatively, Sigma supplies a standard kit that is useful for calculating molecular weights under neutral pH, nondenaturing conditions. The samples and standard proteins, should be used at concentrations of ~1 to 2 μg/μl.

Materials

4× acetic acid gel buffer (200 mM acetic acid, pH 3.7 to 5.6; see recipe)
4× phosphate gel buffer (400 mM sodium phosphate, pH 5.8 to 8.0; see recipe)
4× Tris gel buffer (200 mM Tris·Cl, pH 7.1 to 8.9; see recipe)
4× glycine gel buffer (200 mM glycine, pH 8.6 to 10.6; see recipe)
300 mM sodium sulfite (0.38 g in 10 ml H_2O; used in acetic acid gel preparation)
Protein samples to be analyzed

Contributed by Sean R. Gallagher
Current Protocols in Cell Biology (2000) 6.5.1-6.5.11
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Native protein standards
Electrophoresis buffer: appropriate 4× gel buffer diluted to 1× with H_2O
75-ml side-arm flask (used in gel preparation)

Additional reagents and equipment for gel electrophoresis (*UNIT 6.1*) and staining proteins in gels (*APPENDIX 3*)

Prepare the gel

1. Assemble the glass-plate sandwich of the gel electrophoresis unit and secure it to the casting stand.

   Either single-concentration or gradient gels can be used in the minigel or standard-size format. Gradient gels will enhance the band sharpness of the separated proteins.

2. Prepare acrylamide solutions according to the recipes in Table 6.5.1, Table 6.5.2, Table 6.5.3, or Table 6.5.4, adding the ammonium persulfate and TEMED just before use.

   Deaeration of the solution before the polymerization catalysts are added will speed polymerization by removing inhibitory oxygen, but this is not generally required. The pH used depends on many factors. The most important are the pI values of both the protein of interest and any contaminants, as well as protein mobility and protein solubility. Determining which pH and thus which buffer system to use is largely empirical. However, extremes of pH (<4.0 and >9.0) can lead to denaturation and should be avoided. Prior knowledge of the pI of a protein (UNITS 6.4) allows determination of the net charge under

Table 6.5.1 Recipes for Acetic Acid Nondenaturing Polyacrylamide Gels[a]: pH Range 3.7 to 5.6[b]

Stock solution[c]	Final acrylamide concentration in gel (%)[d]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
300 mM sodium sulfite[e]	0.4	0.4	0.4	0.4	0.4	0.4	0.4
4× acetic acid gel buffer	10	10	10	10	10	10	10
H_2O	22.58	19.28	15.98	12.48	9.28	5.96	2.68
10% (w/v) ammonium persulfate[e,f]	0.3	0.3	0.3	0.3	0.3	0.3	0.3
TEMED[f]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 300 mM sodium sulfite, 4× acetic acid gel buffer (see Reagents and Solutions), and H_2O. If desired to speed polymerization, degas under vacuum ~5 min. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

[b]The pH range can be extended to ~2.0 (the pH of acetic acid) by using unadjusted acetic acid in place of 4× acetic acid gel buffer, although there is little buffering capacity at this pH.

[c]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[d]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

[e]Must be freshly made. Sodium sulfite is needed for efficient polymerization at acid pH.

[f]Added just before polymerization.

Table 6.5.2 Recipes for Phosphate Nondenaturing Polyacrylamide Gels[a]: pH Range 5.8 to 8.0

Stock solution[b]	Final acrylamide concentration in gel (%)[c]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× phosphate gel buffer	10	10	10	10	10	10	10
H_2O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate[d,e]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× phosphate gel buffer (see Reagents and Solutions), and H_2O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.
[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.
[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.
[d]Must be freshly made.
[e]Added just before polymerization.

the separation conditions (i.e., if gel pH < protein pI, the protein will have a net positive charge; if gel pH > protein pI, the protein will be negatively charged).

3. Pour gel to 2 cm from the top of the gel mold and insert the comb. Avoid trapping air bubbles under the comb teeth.

   Air bubbles will cause small semicircular depressions in the well and lead to distortions in the protein banding pattern.

4. Allow gel solution to polymerize 1 to 2 hr.

   Polymerization is indicated by a sharp optical discontinuity around the wells.

Prepare samples and load the wells

5. Solubilize the protein sample to be analyzed using 5% (w/v) sucrose in water or dilute (1 to 5 mM) gel buffer if possible. Also prepare native protein standards.

   The concentration of protein will vary depending on the sample complexity and detection method. For Coomassie blue staining of highly enriched samples such as the standards, use 1 to 2 mg/ml (1 to 2 μg/μl). For more complex mixtures, use 5 to 10 mg/ml (5 to 10 μg/μl). Load 10- to 100-fold less for silver staining. In general, samples should be loaded in a minimum volume, preferably 10 to 20 μl for 0.75- and 1.5-mm-thick gels, respectively. With thin gels, this means using a more concentrated protein sample.

6. Remove comb carefully and rinse wells with electrophoresis buffer (appropriate 4× gel buffer diluted to 1×).

   Rinsing with electrophoresis buffer is needed to remove residual unpolymerized acrylamide monomer, which will continue to polymerize after comb removal, creating uneven wells that may interfere with sample loading.

Table 6.5.3 Recipes for Tris Nondenaturing Polyacrylamide Gels[a]: pH Range 7.1 to 8.9

Stock solution[b]	Final acrylamide concentration in gel (%)[c]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× Tris gel buffer	10	10	10	10	10	10	10
H_2O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate[d,e]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× Tris gel buffer (see Reagents and Solutions), and H_2O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

[d]Must be freshly made.

[e]Added just before polymerization.

7. Fill wells with electrophoresis buffer. If desired, prerun gel.

The gel can be prerun at this point to remove any charged material such as ammonium persulfate from the gel prior to loading the sample. Assemble the electrophoresis unit and fill the buffer chambers with electrophoresis buffer. Run the gel at 300 V until the current no longer drops. This should take ~30 min. Disassemble the unit, discard the buffer, and proceed to the next step.

8. Carefully load up to 10 µl (0.75-mm gels) or 20 µl (1.5-mm gels) sample per lane as a thin layer at the bottom of the wells. Load control wells with native protein standards. Add an equal volume of electrophoresis buffer to any empty wells to prevent spreading of adjoining lanes.

Mobility (R_f) markers require special consideration in nondenaturing gel systems. For cationic systems, cytochrome c (pI ~9 to 10, 5 to 10 µg/lane) works well as an R_f marker. Bromphenol blue (10 µg/ml) is a suitable marker for anionic systems. The marker should be included in the solubilization buffer with the sample.

Perform the separation

9. Assemble the gel unit, fill the upper and lower buffer chambers with electrophoresis buffer, and connect the unit to the power supply. Set current to 30 mA for a 1.5-mm-thick gel (15 mA for a 0.75-mm-thick gel).

If the protein is negatively charged under the separation conditions, then the standard SDS-PAGE electrode polarity should be used (proteins will migrate to the anode or positive electrode; see UNIT 6.1). If the protein is positively charged, then the electrodes should be reversed at the power supply (i.e., red high-voltage cable to the black output and black high-voltage lead to the red output) so the positively charged protein migrates to the negative cathode.

Table 6.5.4 Recipes for Glycine Nondenaturing Polyacrylamide Gels[a]: pH Range 8.6 to 10.6

Stock solution[b]	Final acrylamide concentration in gel (%)[c]						
	5	7.5	10	12.5	15	17.5	20
30% acrylamide/0.8% bisacrylamide	6.7	10	13.3	16.8	20	23.32	26.6
4× glycine gel buffer	10	10	10	10	10	10	10
H_2O	23.08	19.78	16.48	12.98	9.78	6.46	3.18
10% (w/v) ammonium persulfate[d,e]	0.2	0.2	0.2	0.2	0.2	0.2	0.2
TEMED[e]	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Preparation of gel

In a 75-ml side-arm flask, mix 30% acrylamide/0.8% bisacrylamide solution (see Table 6.1.1), 4× glycine gel buffer (see Reagents and Solutions), and H_2O. If desired, degas under vacuum ~5 min to speed polymerization. Add 10% ammonium persulfate and TEMED. Swirl gently to mix. Use immediately.

[a]The recipes produce 40 ml gel solution, which is adequate for one gel of dimensions 1.5 mm × 14 cm × 16 cm or two gels of dimensions 0.75 mm × 14 cm × 16 cm.

[b]All reagents and solutions used in the protocol must be prepared with Milli-Q-purified water or equivalent.

[c]Units of numbers in table body are milliliters. The desired percentage of acrylamide in the gel solution depends on the molecular size of the protein being separated.

[d]Must be freshly made.

[e]Added just before polymerization.

10. Continue electrophoresis until the R_f marker reaches the bottom of the gel.

    For minigels, electrophoresis will take 1 to 2 hr. Standard gels require 4 to 6 hr runs.

11. Turn off power supply, disassemble the unit, and remove gel from sandwich.

12. Stain the gel according to *APPENDIX 3*.

NATIVE DISCONTINUOUS ELECTROPHORESIS AND GENERATION OF MOLECULAR WEIGHT STANDARD CURVES (FERGUSON PLOTS)

ALTERNATE PROTOCOL

One straightforward approach to discontinuous native electrophoresis is to leave out the SDS and reducing agent (DTT) from the standard Laemmli SDS-PAGE protocol (*UNIT 6.1*). The gels are prepared as described in *UNIT 6.5* except that the sample buffer contains no SDS or DTT (samples are not heated), and the gel and electrophoresis solutions are prepared without SDS. This protocol illustrates the separation of standard proteins at four different concentrations of acrylamide and how the results are used to construct a molecular weight standard curve (Ferguson plot) without the need for SDS. By plotting relative mobility against %T (percentage weight per volume of acrylamide plus bisacrylamide in the gel), the presence of isoforms and multimeric proteins can also be detected.

Materials

4× Tris·Cl, pH 8.8 (1.5 M Tris·Cl; *APPENDIX 2A*)
4× Tris·Cl, pH 6.8 (0.5 M Tris·Cl)
Protein sample of interest
2× Tris/glycerol sample buffer (see recipe)

Native protein standards (e.g., Sigma nondenatured protein molecular weight kit)
Tris/glycine electrophoresis buffer (see recipe)

1. Assemble the glass-plate sandwich of the gel electrophoresis unit and place it in the casting stand.
2. Prepare and cast the gels, using 4× Tris·Cl, pH 8.8, for the separating gel and 4× Tris·Cl, pH 6.8, for the stacking gel instead of the SDS-containing counterparts (Table 6.1.1). Prepare a minimum of four separate gels at different acrylamide concentrations.

   A typical range of concentrations is from 5% to 12.5% (e.g., 5%, 7.5%, 10%, 12.5% acrylamide). As with SDS-PAGE, typical gel thickness ranges from 0.75 to 1.5 mm. The 0.75-mm-thick gels are recommended because they offer a combination of fast staining and high resolution.

3. Mix protein sample of interest 1:1 with 2× Tris/glycerol sample buffer to attain a 1 to 2 µg/µl final concentration. Also prepare native protein standards. Remove comb, rinse wells, and load 10 to 20 µl per well for Coomassie brilliant blue staining, and 1 to 2 µl for silver staining.

   Some proteins must be dissolved in 50 mM NaCl or water to become fully solubilized prior to mixing with the sample buffer (Sigma, 1986).

4. Assemble gel electrophoresis unit, using Tris/glycine electrophoresis buffer to fill both lower and upper buffer chambers. Connect power supply and conduct electrophoresis.

   Conditions for separation are the same as for discontinuous SDS-PAGE (i.e., 30 mA for 1.5-mm-thick gels, 15 mA for 0.75-mm-thick gels). For standard-size gels the separation takes 4 to 5 hr; for minigels, 1 to 2 hr is required. Alternatively, standard gels can be run at 4 to 6 mA/gel overnight.

5. After the bromphenol blue R_f marker has reached the bottom of the gel, fix and stain the proteins in the gels according to *APPENDIX 3*. Estimate relative mobilities of the proteins.

   An example of a stained gel is shown in Figure 6.5.1. A minimum of four gel concentrations is recommended. In Figure 6.5.1, Sigma native molecular weight standards were separated on 5%, 7.5%, 10%, and 12.5% acrylamide gels (5.1%, 7.7%, 10.3%, and 12.8% T, respectively).

6. Plot log R_f against gel concentration (% T) (Fig. 6.5.2). Determine the slope of K_r using linear regression.
7. Plot $-\log K_r$ of the curves from step 6 against log molecular weight of the standards (Fig. 6.5.3). Determine the slope using linear regression.
8. Estimate the size of the standards and unknowns from the generated curve (Ferguson plot).

   Use the curve generated by linear regression to estimate the predicted size of the standard for comparison to the actual size stated by the supplier. This indicates the accuracy of the curve. The $-\log K<D_r$ value (y) of the unknown is then used to predict the molecular weight (x).

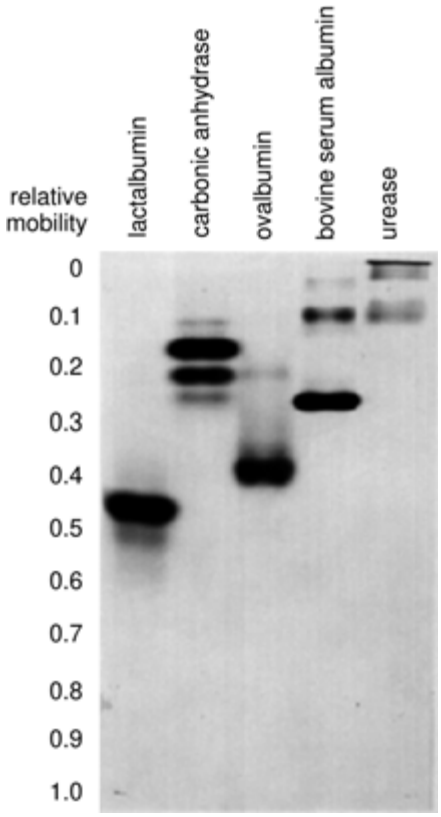

Figure 6.5.1 Separation of native protein standards under nondenaturing conditions by discontinuous polyacrylamide gel electrophoresis at 12.8% T. Approximately 20 μg protein was loaded per lane on a 1.5-mm-thick, 16-cm-long gel. The gel and samples were prepared according to the Alternate Protocol and were electrophoresed 16 hr at 6 mA. Proteins were stained with Coomassie blue.

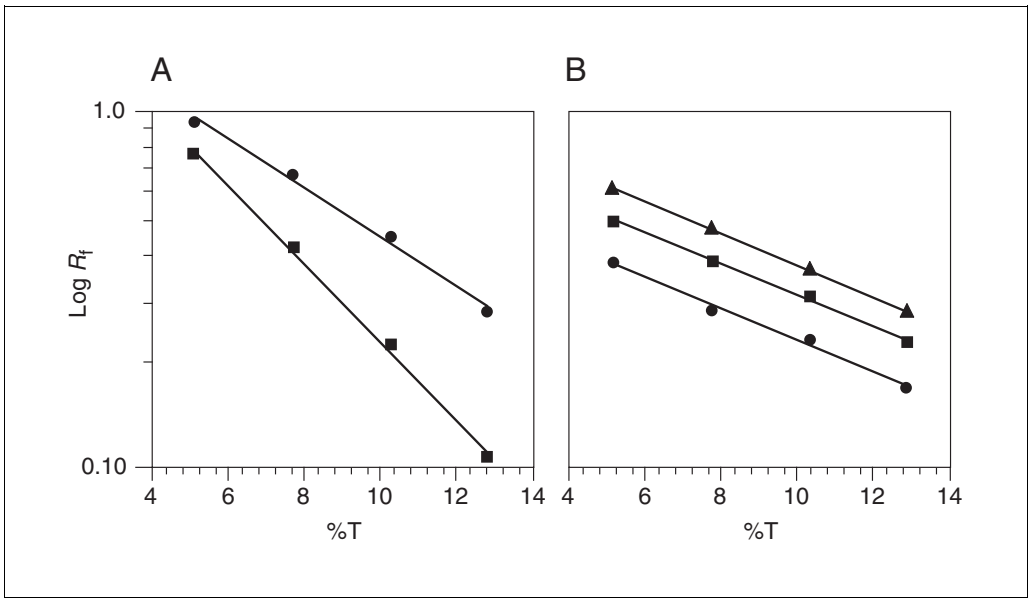

Figure 6.5.2 Effect of %T on the relative mobility of several native proteins. The relative mobility (R_f) of the standard proteins shown in Figure 6.5.1 was determined at four different gel concentrations and plotted as log R_f against %T. See text for details. (**A**) BSA monomer (squares) and dimer (circles); (**B**) carbonic anhydrase isoforms.

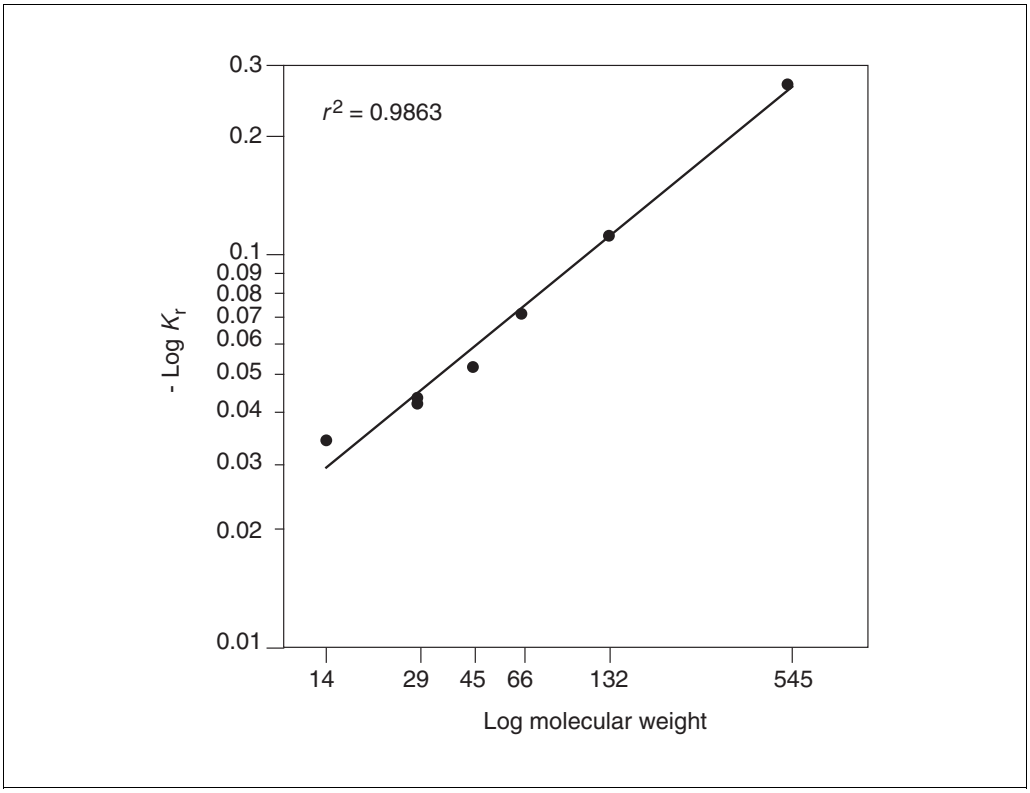

Figure 6.5.3 Native molecular weight standard curve. The –log slope of the line (K_r) from Figure 6.5.2 is plotted against log molecular weight of the standards.

REAGENTS AND SOLUTIONS

*Use Milli-Q-purified water or equivalent for the preparation of all buffers. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Acetic acid gel buffer, 4× (200 mM acetic acid, pH 3.7 to 5.6)

11.49 ml glacial acetic acid
Add to 500 ml H_2O
Adjust to pH 3.7 to 5.6 with 1 M NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Glycine gel buffer, 4× (200 mM glycine, pH 8.6 to 10.6)

15.01 g glycine
Add to 500 ml H_2O
Adjust to pH 8.6 to 10.6 with 1 M NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Phosphate gel buffer, 4× (400 mM sodium phosphate, pH 5.8 to 8.0)

55.2 g $NaH_2PO_4 \cdot H_2O$
Add to 500 ml H_2O
Adjust to pH 5.8 to 8.0 with 1 M NaOH
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Tris gel buffer, 4× (200 mM Tris·Cl, pH 7.1 to 8.9)

24.23 g Tris base
Add to 500 ml H_2O
Adjust to pH 7.1 to 8.9 with 1 M HCl
Add H_2O to 1000 ml
Store up to 1 month at 4°C

Tris/glycerol sample buffer, 2×

25 ml 0.5 M Tris·Cl, pH 6.8 (*APPENDIX 2A*)
20 ml glycerol
1 mg bromphenol blue
Add H_2O to 100 ml and mix
Store in 1-ml aliquots up to 6 months at −70°C

Tris/glycine electrophoresis buffer

15.1 g Tris base
72.0 g glycine
H_2O to 5000 ml
Store up to 1 month at 4°C

COMMENTARY

Background Information

Under nondenaturing conditions, in which protein activity, native charge, and conformation are sustained, electrophoretic separation depends on many factors, including size, shape, and charge. Characteristics such as intrinsic molecular weight (i.e., in the absence of denaturation), the number of isoforms, and the presence of multimeric proteins can be determined with nondenaturing electrophoresis (often called native electrophoresis).

The most important application of nondenaturing electrophoresis is in the determination of native protein size (see Alternate Protocol). Ferguson plots (reviewed by Andrews, 1986) were first described for starch gels (Ferguson, 1964) and then for polyacrylamide gels (Hedrick and Smith, 1968). Ferguson plots are prepared by separating proteins under nondenaturing conditions at several different gel concentrations. As the acrylamide concentration (%T) is increased, the relative mobility (R_f) of the protein decreases. This is plotted as log relative mobility (on the *y* axis) versus %T (on the *x* axis) to produce a straight line. The slope of this line is referred to as the retardation coefficient (K_r) and measures how effectively a protein is slowed by the increase in %T. Large proteins will be retarded much more significantly than small proteins with increasing gel concentration, with the size of the protein being proportional to the slope of the curve. Once the K_r plots for several size standards are generated (Fig. 6.5.2), the K_r values are plotted against the molecular weight of the standard proteins using a log-log graph (Fig. 6.5.3). The retardation coefficient also depends on a large number of other variables including temperature, pH, buffer type, ionic strength, and %C (percent bisacrylamide cross-linker). All these factors should be kept constant for a given experiment.

In addition to estimated size, other types of information are available from the Ferguson plots (Rodbard and Chrambach, 1971; Andrews, 1986). For example, if two components differ in size but have the same charge per unit size (e.g., for a multimeric protein with identical subunits), curves similar to those illustrated by BSA monomer and dimer (Fig. 6.5.2A) will result. Note that when the curve is extrapolated back to 0% T, it is evident that the monomer and the dimer have similar free solution mobilities. Furthermore, as the acrylamide concentration is increased, the separation between the two also increases. However, if two proteins have similar sizes but different amounts of charge, the curves will be parallel on the log plot. This is illustrated by the carbonic anhydrase isoforms (Fig. 6.5.2B). In this example, optimal separation of the isoforms occurs at the lower concentrations of acrylamide as this is a log plot.

Further applications of nondenaturing electrophoresis include preparative purification. The pH of the gel determines the net charge on the protein. Below its isoelectric point (pI) a protein will have a net positive charge, whereas above its pI it will have a net negative charge.

In general, most proteins will be positively charged at pH 2.0 to 4.0; above pH 8.0, most proteins will be negatively charged. As these general guidelines imply, the majority of proteins have isoelectric points between pH 4.0 and 8.0. There are, however, many exceptions. A protein with a highly acidic isoelectric point (e.g., pepsin, with a pI of 2.2) will remain negatively charged at a pH down to its pI. Although a full range of pH options are given, extremes of pH (<4.0 and >9.0) should be avoided, if possible, to minimize denaturation or inactivation. By picking an appropriate electrophoresis pH, it is possible to ensure that the protein of interest will be either positively or negatively charged so that it can be selectively run into the gel, excluding a large proportion of contaminants that have the opposite or no charge. Furthermore, the pH conditions determine the mobility and can be adjusted to ensure a difference in mobility between the protein of interest and contaminants.

Continuous gel systems (see Basic Protocol) offer the most flexibility in terms of separation design. The pH can be tailored so that a given protein has a net positive, neutral, or negative charge. Depending on the polarity of the gel, the protein can then be excluded from or electrophoresed into the gel. Discontinuous gels have a fixed pH and gel polarity. For the nondenaturing Laemmli gel presented in the Alternate Protocol, the proteins of interest should have an isoelectric point of ≤7.0 in order to be negatively charged so that they move into the gel. Other more basic and more acidic discontinuous gel systems can be found in Hames (1990) and Schägger (1994).

Critical Parameters

The success of a gel separation under nondenaturing conditions depends on many factors, and two of the most important are protein solubility and isoelectric point. The protein must be soluble at the pH and the ionic strength of the gel, and it must be charged at that pH in order to move into the gel. If the protein experiences a pH below its isoelectric point, then it will have a net positive charge and will move to the negative electrode. Note that this is the reverse of typical SDS-PAGE. If the protein experiences a pH above its isoelectric point, it will have a net negative charge and will migrate to the positive electrode.

Solubility is a complex issue. Membrane-associated and other hydrophobic proteins are difficult to separate by nondenaturing electrophoresis (Schägger, 1994). Nonionic detergents at concentrations up to 1% and solubilizing reagents such as urea (4 to 8 M) can be used, but these reagents, especially urea, are likely to alter the protein's conformation and most likely the isoelectric point by exposing previously hidden charged groups. If detergent or urea must be included for solubilization, the minimum required to solubilize the protein should be used. Schägger (1994) lists several nonionic detergents suitable for solubilization. Among the more popular are octylglucoside and CHAPS. In general, detergents should be used near the critical micelle concentration (CMC; 0.001% to 1%, depending on the detergent).

The gel concentration has a dramatic effect on resolution and should be optimized in order to achieve the best separation and band sharpness. In general, increasing the %T will improve band sharpness.

Troubleshooting

Gel polymerization at acid pH can be problematic, and sodium sulfite is needed for efficient polymerization (Andrews, 1986). Both the ammonium persulfate and the sodium sulfite must be freshly made, and the highest quality reagents available should be used. Furthermore, the gel solutions should be at room temperature for effective polymerization.

If the protein does not enter the gel and no stained material is present at the well surface, try reversing the polarity of the electrode. If material concentrates at the top of the gel, try lowering the acrylamide concentration. Stained material at the top of the gel may also indicate poor solubilization, and increasing the ionic strength of the solubilization buffer or adding a small amount of urea and/or nonionic detergent may be required.

Anticipated Results

Proteins will resolve depending on their solubility and native charge at the chosen pH. Ideally, a distinct band representing the protein of interest will be visible. If the band is diffuse, then increasing the gel concentration or using a gradient gel will improve resolution. If the band is not visible, then the protein may be at its isoelectric point or may have moved out of the gel because it had the wrong charge. Continuous gel systems, although more versatile, will give lower resolution than discontinuous gels. Detergents or other solubilizing agents such as urea may be needed to fully solubilize and resolve the protein. Once the conditions that resolve the protein are determined, Ferguson plots will give indications of multiple

subunit structure, native size, and potential isoform relationships.

Time Considerations

Separations will be complete when the tracking dye or protein reaches the bottom of the gel. For minigels, this generally takes 1 to 2 hr, using 15 or 30 mA for 0.75- or 1.5-mm-thick gels, respectively. Standard-format gels require 4 to 5 hr at 15 or 30 mA for 0.75- or 1.5-mm-thick gels, respectively. Standard-format gels can also run overnight at 4 to 6 or 8 to 12 mA for 0.75- or 1.5-mm-thick gels, respectively.

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Key Reference

Andrews, 1986. See above.

Covers a variety of electrophoretic techniques, including nondenaturing electrophoresis and Ferguson plots.

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Staining Proteins in Gels

Polyacrylamide gel electrophoresis (PAGE) is a powerful method for the resolution of protein mixtures according to the proteins' charge-to-mass ratio (nondenaturing-PAGE; *UNIT 6.5*), mass (SDS-PAGE; *UNIT 6.1*), isoelectric point (isoelectric focusing) or combinations of these properties (two-dimensional PAGE; *UNIT 6.4*). A number of protocols have been developed to visualize the separated protein bands following electrophoresis. In this unit, four different methods, which are widely used in cell biology laboratories, are described. These are: staining with the dye Coomassie blue (Basic Protocol 1 and Alternate Protocol 1), silver staining (Basic Protocol 2), fluorescent staining with SYPRO Ruby (Basic Protocol 3), and negative staining with zinc (Basic Protocol 4).

Staining with Coomassie blue (Basic Protocol 1) is a relatively simple and very popular method, albeit less sensitive than other procedures. A variation of this protocol, suitable for staining proteins in isoelectric focusing gels, is described under Alternate Protocol 1. Staining with silver (Basic Protocol 2) is probably the most sensitive method, although it is time-consuming and incompatible with some downstream applications (see Commentary). The remaining two methods (Basic Protocols 3 and 4) are more sensitive that Coomassie blue staining and recommended for downstream applications where protein fixation needs to be avoided (e.g., analysis of biological activity). Some of the protocols can be combined. For instance, some researchers prefer to stain gels first with Coomassie blue (Basic Protocol 1) or zinc (Basic Protocol 4) and, if higher sensitivity is necessary, subsequently with silver (Basic Protocol 2). Further details on the differences between the methods, as well as guidelines for their selection according to specific applications, are discussed in the Commentary.

In the following protocols, the volumes of solutions are indicated in "gel volumes." When staining slab mini-gels, 10 gel vol correspond to ~50 to 80 ml.

STAINING PROTEIN GELS WITH COOMASSIE BLUE

BASIC PROTOCOL 1

Coomassie brilliant blue R-250, a triphenylmethane anionic dye, binds avidly to almost all proteins in either native or denatured states and is widely used for detection of proteins in polyacrylamide gels. In this protocol, proteins separated by either nondenaturing- or SDS-PAGE (one- or two-dimensional) are fixed and stained by soaking the gel in a Coomassie blue staining solution; the unbound dye is subsequently removed by washing with destaining solution to yield blue protein bands on a clear background.

Materials

Polyacrylamide gel containing protein(s) of interest (see *UNIT 6.1*)
Coomassie blue staining solution (see recipe)
Destaining solution: 30% (v/v) methanol/10% (v/v) acetic acid in distilled water (store up to 4 months at room temperature)
Storage solution: 7% (v/v) acetic acid/5% (v/v) methanol in distilled water (store up to 4 months at room temperature)
Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
Platform shaker (optional)

CAUTION: Glacial acetic acid and methanol are volatile and toxic. The destaining and storage solutions should be prepared in a chemical fume hood, and gloves should be worn throughout the staining procedure.

Contributed by Esteban C. Dell'Angelica and Juan S. Bonifacino
Current Protocols in Cell Biology (2000) 6.6.1-6.6.14
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NOTE: All steps should be performed at room temperature.

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing a large excess (~10 gel vol) of Coomassie blue staining solution.
2. Incubate with gentle agitation for ≥20 min for a gel ≤1 mm thick or ≥1 hr for a gel >1 mm thick.

   The use of a platform shaker is recommended. Most proteins are fixed due to the presence of methanol in the staining solution; gels can therefore be left in staining solution for many hours (i.e., overnight) without any adverse effect, unless the protein(s) of interest is very small (<5 kDa) and can be lost by diffusion due to incomplete fixation.
3. Remove the Coomassie blue staining solution and rinse the gel briefly with distilled water.

   The Coomassie blue staining solution can be reused several times.
4. Add ~10 gel vol of destaining solution and incubate with gentle agitation until the solution becomes as dark as the gel matrix.
5. Discard the destaining solution.
6. Repeat steps 4 and 5 several times until a clear background is obtained.

   Both the total amount of destaining solution and the destaining time can be reduced by placing a piece of adsorbent material with affinity for the Coomassie dye (e.g., Whatman 3MM filter paper) inside the container having the gel in destaining solution.

   NOTE: *Avoid excessive incubation of the gel with destaining solution as it can result in decreased sensitivity due to dissociation of protein-dye complexes.*
7. Add ~10 gel vol storage solution and incubate for 10 to 15 min with gentle agitation.

   At this point, the gel can be stored at 4°C for several months in a plastic container or sealed plastic bag containing storage solution. Alternatively, it can be soaked in 2% (v/v) glycerol for 15 to 30 min, placed onto Whatman 3MM filter paper, and subsequently dried on a vacuum system.

ALTERNATE PROTOCOL 1

STAINING PROTEIN GELS WITH COOMASSIE BLUE AFTER ISOELECTRIC FOCUSING

In polyacrylamide gel isoelectric focusing, proteins are resolved based on their isoelectric points on a pH gradient generated by a mixture of ampholytes. Many commercially available ampholytes bind Coomassie blue and, therefore, interfere with protein detection. To overcome this problem, the following procedure involves treatment with trichloroacetic acid (TCA) to fix proteins while removing ampholytes and uses $CuSO_4$ in the staining solution to help reduce the background (Righetti and Drysdale, 1974).

Additional Materials *(see also Basic Protocol 1)*

Polyacrylamide isoelectric focusing gel with protein(s) of interest (e.g., *UNITS 6.4 & 15.4*)
20% (w/v) TCA solution in water
IEF Coomassie blue stock solution (see recipe)
10% (w/v) $CuSO_4$ in distilled water (store for up to 4 months at room temperature)

CAUTION: TCA is extremely caustic. Protect eyes and wear gloves when preparing and handling TCA solutions.

1. Make fresh staining solution by mixing 90 ml of destaining solution, 10 ml of IEF Coomassie blue stock solution and 1 ml of 10% (w/v) $CuSO_4$ in distilled water.
2. Remove polyacrylamide gel from isoelectric focusing assembly and place it in a plastic container with lid containing 5 to 10 gel vol of 20% (w/v) TCA solution in water.
3. Incubate for 30 to 60 min with gentle agitation.

   The use of a platform shaker is recommended. In this step, proteins are selectively fixed while ampholytes are removed from the gel.
4. Remove the TCA solution and add ~10 gel vol destaining solution.

   Do not reuse the TCA solution; dispose of it following applicable safety regulations for chemical waste. In this step, the TCA is removed from the gel.
5. Incubate 15 min with gentle agitation.
6. Discard the destaining solution and add freshly prepared staining solution (from step 1).
7. Incubate with gentle agitation for ≥20 min for a gel ≤1 mm thick or ≥1 hr for a gel >1 mm thick.
8. Remove the staining solution and rinse the gel briefly with distilled water.

   Do not reuse the staining solution.
9. Destain the gel (see Basic Protocol 1, steps 4 to 7).

STAINING PROTEIN GELS WITH SILVER

BASIC PROTOCOL 2

Silver staining is based on the selective reduction of silver ions at sites of the gel that contain proteins and other macromolecules. The following protocol is adapted from that of Blum et al. (1987), and involves pretreatment of a fixed polyacrylamide gel with thiosulfate (step 6) followed by impregnation with silver nitrate (step 9) and color development with formaldehyde at a high pH (steps 12 and 13). Pretreatment with thiosulfate significantly increases the sensitivity and improves the contrast of protein staining. In addition, the presence of low concentrations of thiosulfate during color development reduces nonspecific staining of the gel surface. The procedure is applicable for both nondenaturing- (*UNIT 6.5*) and SDS-PAGE (one- or two-dimensional; *UNIT 6.1* and *UNIT 6.4*) and yields dark (brown to black) protein bands on a clear background.

Materials

Polyacrylamide gel containing protein(s) of interest (see *UNITS 6.1, 6.4 & 6.5*), either unfixed or fixed and stained with Coomassie blue (see Basic Protocol 1 and Alternate Protocol 1)
Deionized water (HPLC grade or Milli-Q)
50% (v/v) ethanol in deionized water
Fixative solution (see recipe)
Thiosulfate solution (see recipe)
Silver nitrate solution (see recipe)
Developer solution (see recipe)
50% (v/v) methanol/12% (v/v) acetic acid in deionized water
50% (v/v) methanol in water

continued

Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
Clean plastic containers (at least six; lids of pipet tip containers are appropriate for staining mini-gels)
Aluminum foil
Platform shaker (optional)

CAUTION: Glacial acetic acid and methanol are volatile and toxic; solutions containing these solvents should be prepared in a chemical fume hood and handled with care.

NOTE: All solutions should be prepared in deionized water (Milli-Q or HPLC grade). At all times wear gloves that have been rinsed extensively with distilled water. Handle the gel by using clean forceps having blunt tips or by touching the corners with clean, powder-free gloves. All steps should be performed at room temperature.

Fix the gel

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing a large excess (~10 gel vol) of fixative solution.

   Steps 1 and 2 are for unfixed gels only. If the polyacrylamide gel was previously fixed and/or stained with Coomassie blue (Basic Protocol 1 or Alternative Protocol 1), proceed directly to step 3.

2. Incubate for ≥30 min with gentle agitation.

   The use of a platform shaker is recommended.

Pretreat the gel

3. Transfer the gel to a clean plastic container having ~10 gel vol of 50% ethanol in deionized water. Incubate 20 min with gentle agitation.
4. Replace the solution with another ~10 gel vol of 50% (v/v) ethanol in deionized water. Incubate 20 min with gentle agitation. Repeat this step once (total of three pretreatment steps).

   The plastic container used in steps 3 and 4 can be reused in step 16. Discard the ethanol solution and rinse the container briefly with deionized water.

5. Transfer the gel to a clean plastic container having ~10 gel vol deionized water and incubate 5 to 10 min with gentle agitation.
6. Discard the water and add ~10 gel vol thiosulfate solution. Incubate for exactly 1 min with gentle agitation.

   Incubations longer than 1 min will result in increased background staining.

7. Tranfer the gel to a clean plastic container having ~10 gel vol deionized water and incubate exactly 20 sec.
8. Change the water in the container and incubate exactly 20 sec. Perform this step twice (for a total of three).

   The plastic container used in steps 7 and 8 can be reused in steps 10 and 11 but should first be rinsed thoroughly with deionized water.

Stain the gel

9. Transfer the gel to a clean plastic container containing 5 to 10 gel vol silver nitrate solution. Cover the container with aluminum foil and incubate 20 min with gentle agitation.

   Light exposure causes the reduction of silver ions and may therefore result in increased background staining.

10. Transfer the gel to a clean plastic container having ~10 gel vol deionized water and incubate exactly 20 sec.

11. Change the water in the container and incubate again exactly 20 sec.

The plastic container used in steps 10 and 11 can be reused in steps 14 and 15 but should first be rinsed with deionized water.

Develop the gel stain

12. Transfer the gel to a clean plastic container having ~10 gel vol developer solution.

13. Incubate with gentle agitation until the protein band(s) of interest becomes visible (or until the gel matrix begins to get too dark).

This step lasts a few minutes (≤10 min) and requires continuous visual inspection as the protein bands may develop rapidly, and long incubations may result in high background staining. Since some developing will still occur during steps 14 and 15, it is advisable to proceed to the next step as soon as the staining is judged to be optimal.

14. Transfer the gel to a clean plastic container having ~10 gel vol deionized water. Incubate 30 sec.

15. Change the water in the container and incubate an additional 30 sec.

16. Transfer the gel to a clean plastic container having ~10 gel vol 50% methanol/12% acetic acid in deionized water. Incubate 10 min with gentle agitation.

17. Remove the solution from the container and add ~10 gel vol of 50% methanol.

The gel can be stored in this solution for several months at 4°C. Alternatively, the gel can be rehydrated by soaking in water for 5 to 10 min, soaked in 2% (v/v) glycerol for another 15 to 30 min, placed onto Whatman 3MM filter paper, and then dried on a vacuum system.

FLUORESCENCE DETECTION OF PROTEINS IN GELS

BASIC PROTOCOL 3

In this method, proteins separated on polyacrylamide gels [either nondenaturing- (*UNIT 6.5*) or SDS-PAGE, one- or two-dimensional (*UNIT 6.1* or *UNIT 6.4*)] are incubated with SYPRO Ruby, a commercially available fluorescent compound that interacts specifically with proteins. Following incubation, protein bands can be readily visualized using standard 300-nm transillumination.

Materials

Polyacrylamide gel containing protein(s) of interest (see *UNIT 6.1*, *UNIT 6.4*, or *UNIT 6.5*)
SYPRO Ruby protein gel stain (Molecular Probes)
Distilled water

Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
Platform shaker (optional)
300-nm UV transilluminator
Photographic camera or CCD camera (optional)

CAUTION: The potential toxicity of the SYPRO Ruby dye, which comprises an organic component and ruthenium, has not been fully evaluated. Gloves should be worn throughout the staining procedure. For disposal, the stain solution should be poured through activated charcoal, and the dye adsorbed to activated charcoal destroyed in a chemical incinerator following local environmental regulations.

NOTE: Protect SYPRO Ruby protein gel stain from light. If the plastic container with lid used for staining is transparent, cover it completely with aluminum foil.

NOTE: All steps should be performed at room temperature.

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing a large excess (~10 gel vol) of SYPRO Ruby protein gel stain.
2. Incubate 3 hr with gentle agitation.

   The use of a platform shaker is recommended.
3. Remove the SYPRO Ruby protein gel stain and rinse the gel briefly with distilled water.

   Do not reuse the stain solution. Follow local recommendations for the disposal of potentially toxic organic compounds.
4. Add ~10 gel vol distilled water and incubate 10 min with gentle agitation.
5. Change the distilled water in the container and incubate another 10 min with gentle agitation.
6. Visualize protein bands by fluorescence on a 300-nm UV transillumination unit.

   Images can be acquired using a photographic camera with appropriate film (e.g., Polaroid 667 black-and-white print film) or a CCD camera.

   CAUTION: *Use UV-rated eye protection to avoid direct exposure of the eyes to the UV light.*

   At this point, the gel is ready for downstream applications (e.g., electroelution and immunoblotting).

BASIC PROTOCOL 4

REVERSIBLE PROTEIN STAINING WITH ZINC

In this method, protein bands separated on SDS-PAGE gels [one- or two-dimensional (*UNIT 6.1* or *UNIT 6.4*)] are visualized as clear spots on an opaque white background, the latter being generated by precipitation of SDS with zinc ions. This negative staining procedure is sensitive, fast, and completely reversible upon removal of zinc with a chelating agent.

Materials

Polyacrylamide gel containing protein(s) of interest (see *UNIT 6.1*)
GelCode E-Zinc Reversible Stain Kit (Pierce), containing:
  E-Zinc Stain
  E-Zinc Developer
  E-Zinc Eraser
Distilled water
Plastic container with lid (pipet tip containers are appropriate for staining mini-gels)
Platform shaker (optional)

NOTE: All steps should be performed at room temperature.

Stain the gel

1. Remove polyacrylamide gel from electrophoresis assembly and place it in a plastic container with lid containing ~10 gel vol E-Zinc Stain solution.
2. Incubate 10 min with gentle agitation.

   The use of a platform shaker is recommended.

3. Remove the E-Zinc Stain solution from the container and replace it with ~10 gel vol E-Zinc Developer solution.

   Do not reuse the stain solution.

4. Incubate 1 to 2 min with gentle agitation. Proceed to the next step as soon as the staining is judged to be optimal.

5. Remove the E-Zinc Developer solution from the container and replace it with ~10 gel vol distilled water.

   Do not reuse the developer solution.

Wash the gel

6. Incubate 1 min with gentle agitation.

7. Change the water in the container.

8. Visualize the protein bands by placing the polyacrylamide gel on a dark (black or blue) surface.

   The stained gel can be kept in water for several weeks at 4°C.

Solubilize the SDS/zinc precipitate (optional)

9. Incubate the gel with ~10 gel vol E-Zinc Eraser with gentle agitation until the gel matrix is completely clear (~5 to 10 min).

   These two steps involve solubilization of the SDS/zinc precipitate to completely destain the gel, thus allowing further analysis of the protein band(s) of interest.

10. Rinse the gel with distilled water.

    At this point, the gel is ready for downstream applications (e.g., electroelution, immunoblotting, etc.).

REAGENTS AND SOLUTIONS

*Use HPLC-grade or Milli-Q-purified water or equivalent in all recipes and protocol steps. For common stock solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Coomassie blue staining solution

For 100 ml: Dissolve 0.1 g Coomassie brilliant blue R-250 in a mixture of 30 ml methanol and 20 ml water. Subsequently, add 40 ml of water and 10 ml of glacial acetic acid. Store for up to 4 months at room temperature.

CAUTION: *Glacial acetic acid and methanol are volatile and toxic and should be handled in a chemical fume hood.*

This solution is 0.1% (w/v) Coomassie brilliant blue R-250 in methanol/acetic acid/water (3:1:6 v/v/v).

Developer solution

Dissolve 6 g Na_2CO_3 in 98 ml water. Add 50 µl of 37% formaldehyde and 2 ml of thiosulfate solution (see recipe). Prepare fresh before use.

CAUTION: *Formaldehyde is toxic and carcinogenic. Wear gloves and handle the concentrated (37%) solution in a chemical hood.*

Fixative solution

Mix 50 ml of methanol with 12 ml glacial acetic acid and add water to 100 ml. Add 50 µl of 37% formaldehyde. Prepare fresh before use.

CAUTION: *Glacial acetic acid and methanol are volatile and toxic. Formaldehyde is toxic and carcinogenic. Wear gloves and prepare the solution in a chemical fume hood.*

IEF Coomassie blue stock solution

Dissolve 0.2% (w/v) Coomassie brilliant blue R-250 and 60% (v/v) methanol in water. Store for up to 4 months at room temperature.

Silver nitrate solution

Dissolve 0.2 g $AgNO_3$ in 100 ml water. Add 75 µl of 37% formaldehyde. Protect the solution from light. Prepare fresh before use.

CAUTION: *Formaldehyde is toxic and carcinogenic. Wear gloves and handle the concentrated (37%) solution in a chemical fume hood.*

Discard the solution if it becomes cloudy.

Thiosulfate solution

Dissolve 20 mg $Na_2SO_4 \cdot 5H_2O$ in 100 ml water. Prepare fresh before use.

COMMENTARY

Background Information

The first protocols developed for the visualization of proteins bands after electrophoresis in polyacrylamide or other gel matrices relied on the use of dyes with selective avidity for polypeptides. Among these dyes, which included amido black 10B and fast green FCF, the Coomassie blue dyes gave higher sensitivity (Wilson, 1979) and became routinely used. A large number of staining protocols using Coomassie brilliant blue R-250 as a dye have been published (see Neuhoff et al., 1985, and references therein). The protocol described in this unit (Basic Protocol 1) involves simultaneous protein fixation and staining in a solution containing the dye in methanol/acetic acid/water, followed by removal of the unbound dye by washing with methanol/acetic acid/water. This is the most commonly used protocol for protein staining in gels. Proposed variations to this procedure include using no methanol to retain small polypeptides (Schägger and von Jagow, 1987), decreasing the concentrations of both methanol and acetic acid to limit protein fixation (Rosenfeld et al., 1992), and using ion-pairing agents to reduce background (Choi et al., 1996). Protocols using a colloidal form of Coomassie brilliant blue G-250, which rival in sensitivity those using Coomassie brilliant blue R-250, have also been described (see for example: Neuhoff et al., 1985; DeSilva, 1995).

A dramatic increase in sensitivity was achieved with the introduction of protein staining methods based on the selective reduction of silver ions to form metallic silver images (Switzer et al., 1979). The method relies on the autocatalytic reduction of silver, a key phenomenon of the photographic process, and differences in reduction potential of silver at the sites occupied by macromolecules relative to that at the rest of the gel matrix. The differences in reduction potentials can be manipulated to obtain either a positive or negative image of protein bands. Positive silver staining protocols comprise the following stages: (1) fixation of proteins and elimination of interfering substances (e.g., amino acids, Tris, SDS), (2) sensitization with an agent to increase sensitivity and/or contrast, (3) impregnation with silver solution (silver nitrate, basic silver ammonia or silver-diamine complex), (4) controlled rinse to remove silver ions not associated with proteins, (5) image development, and (6) image development stopping (reviewed by Rabilloud et al., 1994). The procedure described in Basic Protocol 2 (Blum et al., 1987) involves sensitization with thiosulfate, impregnation with silver nitrate, and image development using a dilute formaldehyde solution at high pH. Small amounts of thiosulfate included in the developer solution help to keep the background low by complexing silver ions that otherwise could form spurious silver deposits in the gel matrix. This procedure is highly sensitive and compatible with virtually all types of PAGE systems. Unlike other silver staining protocols, which yield a mixture of positive and negative bands or different band colors, the images of the protein bands are rather uniform. A drawback of this and other silver staining methods is that they can also stain other macromolecules, namely DNA, RNA and bacterial lipopolysaccharides.

Procedures for the fluorescent staining of proteins in polyacrylamide gels include covalent modification with fluorescamine or fluorescein isothiocyanate, and noncovalent staining with the hydrophobic probes 1-anilinonaphthalene-8-sulfonic acid or nile red (see

Steinberg et al., 1996, and references therein). A family of fluorescent protein stain reagents, including SYPRO Orange, SYPRO Red, and SYPRO Ruby, has recently been introduced and reported to be highly sensitive and protein specific (Steinberg et al., 1996). These reagents detect protein-SDS complexes, rather than protein functional groups, and are compatible with most downstream applications. Known interferences are colored stains or prosthetic groups, Triton X-100, and the excess of SDS at the migration front. A simple protocol for protein staining with SYPRO Ruby, which is commercially available and said to be the most sensitive dye within the SYPRO family, is described in Basic Protocol 3.

A different group of protein staining methods for SDS-PAGE are based on the reversible formation of SDS precipitates, thus allowing visualization of protein bands as clear areas on an opaque background. Although SDS precipitation can be simply achieved by lowering the temperature of the gel to 0° to 4°C for a few hours, faster and more sensitive methods involve the formation of insoluble complexes with heavy metal salts such as copper (Garfin, 1990) or zinc (Fernández-Patron et al., 1995a). A protocol for rapid, reversible staining with zinc is described in Basic Protocol 4. The protocol uses a kit that is commercially available at a reasonable price. If cost is a major issue, however, the reader is referred to the protocol described by Fernández-Patron et al. (1995a).

Additional staining methods not discussed in this unit include radiolabeling with [^{35}S]thiourea and silver bromide (Wallace and Saluz, 1992), combined Coomassie blue/zinc staining (Fernández-Patron et al., 1995b) and mixed Evans blue/rhodamine-B staining (Na et al., 1994).

Critical Parameters

Comparison of staining procedures

None of the staining methods is optimal for all types of applications. The choice of the most appropriate method for a particular experiment will depend on a number of considerations, including: sensitivity, simplicity, duration, long-term documentation, quantitation, and downstream applications. The following guidelines are restricted to the procedures described in this unit, namely staining with Coomassie blue (Basic Protocol 1 and Alternate Protocol 1), silver (Basic Protocol 2), SYPRO Ruby (Basic Protocol 3) and zinc (Basic Protocol 4).

Sensitivity

Figure 6.6.1 shows a comparative experiment in which serial dilutions of protein standards were run on SDS-PAGE (*UNIT 6.1*) and subsequently stained following the four Basic Protocols described in this unit. Among the four methods, silver (Fig. 6.6.1B) and Coomassie blue staining (Fig. 6.6.1A) gave the highest and lowest sensitivity, respectively. The detection limit of fluorescent staining with SYPRO Ruby could be greatly enhanced by the integration effect of a CCD or photographic camera. Thus, while by direct observation (on a UV transillumination unit) SYPRO Ruby staining was significantly less sensitive than negative zinc staining, both methods gave comparable sensitivities upon the use of a CCD camera to integrate the signal of SYPRO Ruby-stained bands (Fig. 6.6.1C and D).

Simplicity

While staining with Coomassie blue, SYPRO Ruby, or zinc are relatively simple methods, silver staining involves a number of steps that need to be carefully controlled, most notably sensitization with thiosulfate (Basic Protocol 2, step 6), rinsing with water before and after silver impregnation (Basic Protocol 2, steps 7 to 8 and 10 to 11, respectively) and image development (Basic Protocol 2, step 13). In fact, the duration of the image development step critically influences the sensitivity of silver staining, thus resulting in experiment-to-experiment variations in band color intensity. Staining with SYPRO Ruby requires a UV transilluminator to visualize the protein bands and the careful disposal of this potentially toxic dye (see Basic Protocol 3).

Documentation

Gels stained with Coomassie blue, silver, or zinc can be stored at 4°C for several weeks without significant decrease in sensitivity. For long-term storage, gels stained with Coomassie blue, silver, or SYPRO Ruby can be equilibrated in 2% (w/v) glycerol and dried on a gel dryer, although in the case of SYPRO Ruby–stained gels this can result in decreased sensitivity. For photography or digital image acquisition, the blue bands of Coomassie blue-stained gels have less contrast than the dark brown/black bands of silver-stained gels, and SYPRO Ruby–stained gels require the use of an appropriate photographic film (e.g., Polaroid 667 black and white film) or a sensitive CCD camera. Negatively zinc-stained gels can

be problematic for photography or imaging on a CCD camera.

Quantitative analysis

Scanning densitometric analyses of protein bands from stained polyacrylamide gels have been used to determine the relative abundance of proteins in complex mixtures, the purity of protein samples, and even the stoichiometry of multisubunit protein complexes. These quantitative analyses are performed assuming identical staining properties of the different proteins in the sample (i.e., that their staining results in the same band intensity per protein mass unit). This assumption is not necessarily correct, especially for Coomassie blue or silver staining where band intensities are known to be influenced by the proteins' amino acid compositions. Another limitation is that measurements should be made only within a linear range. Staining with SYPRO dyes, which interact with the protein-SDS complex rather than protein functional groups, has been reported to give relatively less protein-to-protein variability and

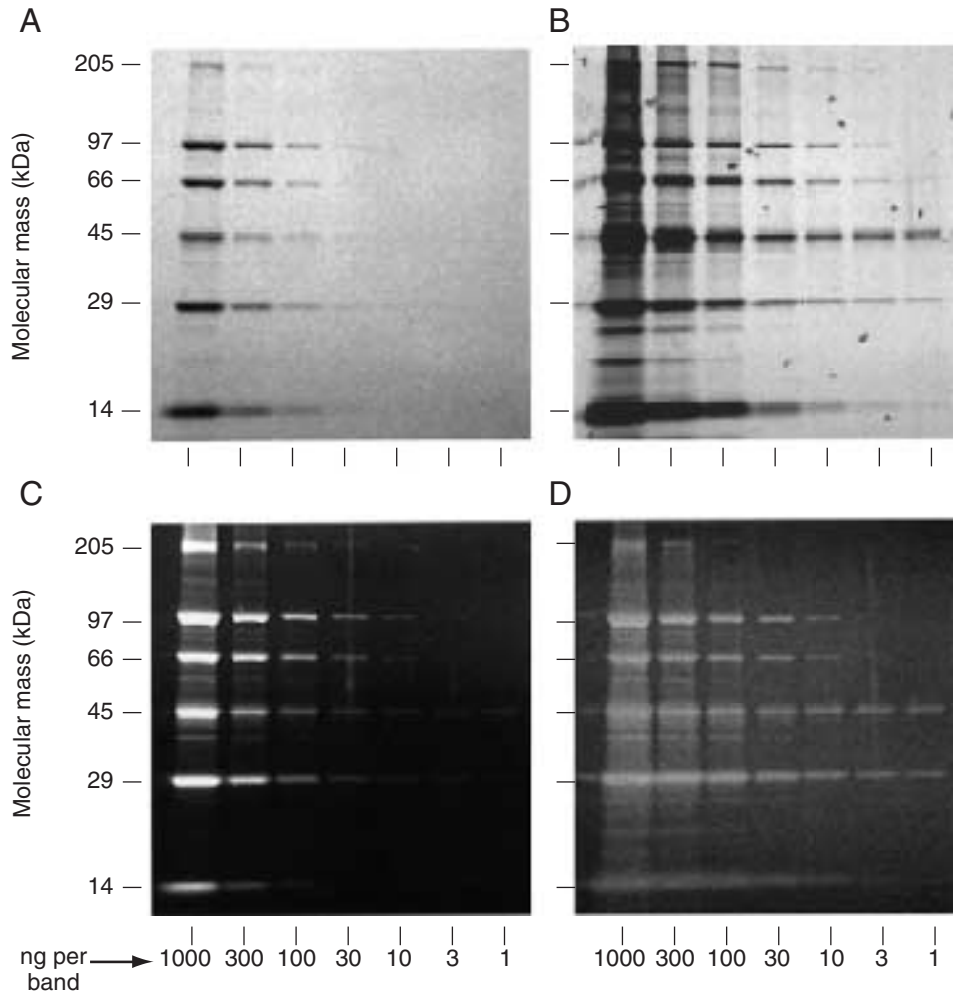

Figure 6.6.1 Comparison of the protein staining methods described in this unit. Serial dilutions of a mixture of rabbit muscle myosin (~205 kDa), rabbit muscle phosphorylase b (~97 kDa), bovine serum albumin (~66 kDa), chicken ovalbumin (~45 kDa), bovine erythrocyte carbonic anhydrase (~29 kDa) and chicken egg lysozyme (~14 kDa) were resolved by SDS-PAGE on a 4% to 20% T gradient gel and stained with: (**A**) Coomassie blue (Basic Protocol 1), (**B**) silver (Basic Protocol 2), (**C**) SYPRO Ruby (Basic Protocol 3), or (**D**) zinc (Basic Protocol 4). The amounts (in nanograms) per band loaded on each lane are indicated at the bottom of the figure. Images of stained gels were acquired on a Bio-Rad Gel Doc 1000 gel documentation system, using a standard UV (300 nm) transilluminator with (**A**, **B**, **D**) or without (**C**) a UV/white light conversion screen (Bio-Rad). The image shown in (**C**) was acquired using an integration time of 0.6 sec in order to optimize sensitivity.

Table 6.6.1 Troubleshooting Guide for Staining with Coomassie Blue (Basic Protocol 1)

Problem	Cause	Solution
No bands are detected		
Background is completely clear	Amount of protein(s) below detection limit	Check protein concentration in original sample. Stain the gel with silver (Basic Protocol 2).
Background is dark blue	Insufficient destaining	Continue to destain the gel until the background is clear (steps 4 to 6).
Protein bands are too faint		
	Staining solution is too old (i.e., methanol has evaporated)	Prepare new Coomassie blue staining solution.
	Insufficient staining time	Re-stain the gel using a longer staining time (step 2)
	Excessive destaining	Re-stain the gel monitoring band and background color during destaining (steps 4 to 6)
High background areas		
High background restricted to lanes with samples	Interfering compounds in the samples	Fix proteins with TCA before staining (see Alternate Protocol 1, steps 2 to 5).
Blue spots at the gel surface	Powder or dirt deposited on the surface during handling of the gel	Use clean, powder-free gloves. Remove powder or dirt by gently touching the surface with a powder-free glove.

Table 6.6.2 Troubleshooting Guide for Staining with Silver (Basic Protocol 2)

Problem	Cause	Solution
Protein bands are absent or too faint		
Background is clear	Amount of protein(s) below detection limit	Check protein concentration in original sample.
	Insufficient image development	Re-stain the gel extending the image development step (step 13) until background begins to get too dark.
	Formaldehyde is too old (white polymers in concentrated solution)	Use formaldehyde from a fresh concentrated solution.
	Silver nitrate solution is not fresh	Prepare fresh silver nitrate solution just before use. Discard the solution if it becomes cloudy.
Background is too dark	Gel has been overdeveloped	Monitor image development (step 13) continuously. Proceed to the next step as soon as the staining is considered satisfactory.
	Poor quality of reagents	Use reagents of the highest purity available. Prepare the solutions using HPLC-grade water.
Band images and background color develop too fast	Concentration of sodium thiosulfate ($Na_2S_2O_3$) in developer and thiosulfate solutions is not optimal	Increase sodium thiosulfate concentration in both solutions (e.g., 2- to 3-fold). Do not reuse the solutions.
Metallic silver is deposited on the gel surface	Powder or dirt deposited on the surface during gel handling	Handle the gels with clean forceps or gloves that have been rinsed with water.
	Metallic silver present in the silver nitrate solution	Protect the solution from light. Discard the solution if it gets cloudly.

Table 6.6.3 Troubleshooting Guide for Fluorescent Staining (Basic Protocol 3)

Problem	Cause	Solution
Protein bands are absent or too faint		
Clear background	Amount of protein(s) below detection limit	Check protein concentration in original sample. Stain the gel with silver (Basic Protocol 2).
	Direct eye visualization is not sensitive enough	Visualize the bands by using a CCD camera or by photography.
	Photographic or imaging conditions are not appropiate	Use sensitive photographic films (Polaroid 667 black-and-white or similar film) or appropiate CCD camera. Increase exposure (integration) time.
	Stain solution too old or inactivated by light	Use fresh stain solution. Protect it from the light. Do not reuse it.
	Protein(s) of interest pre-stained or containing colored prosthetic groups	If possible, remove prosthetic group(s) from proteins and avoid using pre-stained proteins. Otherwise, use an alternative staining method (e.g., Basic Protocol 2 or Basic Protocol 4).
Highly fluorescent background	Too much unbound dye remains in the gel matrix	Rinse the gel further with water (i.e., repeat steps 4 and 5).
	Gel is placed for visualization on an autofluorescent plastic surface (e.g., Saran Wrap)	Place the gel directly onto the surface of the UV transillumination unit.
	Gel is attached to a polyester surface with affinity for the dye (e.g., backing material of PhastGels)	Remove the gel from the polyester surface before visualization.
Heavy stain at the SDS-PAGE migration front	SYPRO dyes bind micellar SDS at the migration front	Allow the SDS front to migrate out of the gel.
Odd marks at the gel surface	Portions of the gel squeezed or have powder deposits due to handling	Handle the gel gently, wearing gloves that have been rinsed with water.

Table 6.6.4 Troubleshooting Guide for Staining with Zinc (Basic Protocol 4)

Problem	Cause	Solution
Protein bands are absent or too faint	Insufficient contrast due to the use of clear surfaces for visualization	Visualize the bands by placing the gel against a dark surface.
	Gel is overdeveloped	Destain the gel (steps 9 and 10) and stain again monitoring the appearance of bands against a dark surface.
	Amount of protein(s) below detection limit	Check protein concentration in original sample. Stain the gel with silver (Basic Protocol 2).
Blackground does not turn opaque	Stain solution or developer are too old	Use fresh stain and developer solutions (do not reuse them).

longer linear ranges than the other staining methods (Steinberg et al., 1996). Nevertheless, in order to check the validity of the measurements it is always advisable to load in the gel, together with the sample(s) to be quantitated, internal controls or a calibration curve with standard protein(s).

Downstream applications

Although PAGE was initially used only for analytical purposes, a large variety of microtechniques have been developed that use PAGE as a preparative step. Proteins separated by one- or two-dimensional PAGE can be treated in-gel with proteases (Rosenfeld et al., 1992) or cyanogen bromide (Córdoba et al., 1997) to obtain peptide fragments for microsequencing, electroeluted, or transferred to nitrocellulose or poly(vinylidene difluoride) (PVDF) membranes (*UNIT 6.2*) for further analysis such as immunodetection or Edman degradation. Proteins separated by nondenaturing PAGE (*UNIT 6.5*), or sometimes even by SDS-PAGE, can be analyzed in-gel for enzymatic or ligand-binding activity. Both the SYPRO Ruby and negative zinc staining methods are compatible with virtually all of these applications. A notable exception is activity analysis of proteins separated by nondenaturing PAGE which are sensitive to SDS, as both staining procedures require the presence of SDS for band visualization. On the other hand, Coomassie blue and silver gel staining involve protein fixation and, therefore, are compatible with fewer downstream applications. It is worth mentioning, however, that Coomassie-stained proteins can be efficiently electrotransferred to PVDF membranes for Edman degradation sequencing (U. Hellman, pers. commun.), although for immunoblot analysis the Coomassie blue dye is a known interference. Coomassie blue staining has been widely used to visualize protein bands destined for in-gel generation of peptides for microsequencing, mainly because these downstream applications have not been sensitive enough to allow analysis of protein bands below the detection limit of the dye. Recent methodological improvements, however, have made it possible to obtain sequence information from as little as 5 ng of protein from polyacrylamide gels stained with silver (Wilm et al., 1996).

Troubleshooting

Tables 6.6.1 to 6.6.4 summarize common problems that may arise during staining of proteins in polyacrylamide gels. Before using any of the methods to analyze a "real" sample, it is advisable to test the method first on a gel containing known amounts of standard proteins (e.g., see Fig. 6.6.1).

Anticipated Results

The protocols described in this unit should allow detection of submicrogram amounts of protein separated by PAGE as exemplified by Figure 6.6.1.

Time Considerations

By far, negative staining with zinc is the fastest method among the ones described in this unit. Images can be obtained within 20 min after the polyacrylamide gel is removed from the electrophoresis unit. Silver staining requires 30 min for fixation and another ~2.5 hr to complete the procedure. Both the Coomassie blue and SYPRO Ruby procedures require ~3.5 hr for obtaining optimal protein-to-background staining ratio. Suboptimal staining with SYPRO Ruby can be obtained by incubating the gel with stain solution for 60 to 90 min and skipping the subsequent washing steps (Basic Protocol 3, steps 4 and 5). In the case of Coomassie blue staining, binding of the dye to proteins is a fast process that depends mainly on its rate of diffusion into the gel matrix (Hitchman and Ekstrom, 1994). Removal of the unbound Coomassie dye from the gel, which also relies on diffusion, can be accelerated by placing an adsorbent material with affinity for the dye (e.g., Whatman 3MM filter paper) into the destaining solution with the gel or by increasing the temperature of destaining (e.g., to 37°C). In both cases, however, caution should be taken to avoid excessive destaining that could decrease sensitivity.

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Agarose Gel Electrophoresis of Proteins

This unit describes the use of agarose gel as a matrix for the electrophoresis of proteins. Although agarose is widely used as a material for molecular sieving, it is not often used for the electrophoresis of proteins. When it is used for this purpose, it is generally employed for the electrophoresis of very large proteins. In other electrophoresis procedures it is part of a composite polyacrylamide/agarose system. The system described below utilizes agarose alone as the gel matrix. As with acrylamide gels, the proteins thus separated may be transferred to a membrane (immunoblotting) for further analysis, or the proteins may be identified directly in the gel using stains or labeled antibodies. A particular advantage of the method described in the Basic Protocol is the preparation of the gel in a horizontal electrophoresis bed which saves time and eliminates many of the difficulties commonly encountered in the preparation of acrylamide or composite gels in a vertical preparation apparatus.

These protocols describe methods for the separation and identification of von Willebrand factor (vWF), an extremely large plasma protein that is comprised of multimers ranging from 850,000 to 20,000,000 Da. The methods can be applied to other mixtures containing large proteins, multimeric proteins, and other large protein complexes such as fibrinogen and fibrin complexes (Shainoff, 1991). While the use of an SDS buffer system allows separation of proteins on the basis of size, nondenatured proteins can be separated in "native" agarose gels if their charge and configuration allow for satisfactory partitioning. Von Willebrand factor multimers are stable in SDS due to their disulfide linkages, and the protocols below utilize SDS. The agarose gel electrophoresis and blotting with immunodetection procedure (see Basic Protocol) utilizes identification of protein by a specific antibody followed by chemiluminescent detection methods. Major advantages of this method include the technical ease of preparing the gel, increased sensitivity, and a much shorter turn-around time than in-gel antibody analysis (see Alternate Protocol). It also eliminates the use of radioactive isotopes**.** In addition, the primary and secondary antibodies can be easily removed and the membrane (PVDF) probed again with a different detection antibody. This allows the laboratory the opportunity to detect a second protein or antigenic site on the same protein and also a "second chance" to correct an omission in the procedure, especially for samples in limited supply (*UNIT 6.2*); however, the immunoblotting step of such large proteins must be done with care in order to assure the adequate transfer of the largest proteins. The Alternate Protocol uses direct identification of the protein in the gel by a specific radiolabeled antibody, eliminating the immunoblotting step, but sacrificing sensitivity.

AGAROSE GEL ELECTROPHORESIS AND BLOTTING WITH IMMUNODETECTION

BASIC PROTOCOL

The following is a method for separating a complex mixture of human plasma proteins by continuous SDS horizontal (submerged) agarose gel electrophoresis. The very large multimers of circulating plasma von Willebrand factor (or more highly purified preparations of this protein) are identified using a specific antibody and visualized using a chemiluminescent reagent. This protocol utilizes a 20×25–cm horizontal gel apparatus. Dry agarose is weighed, mixed with electrophoresis buffer, and melted in a hot water bath. The agarose is allowed to partially cool, then is poured into a horizontal casting frame with a Teflon comb in place, and allowed to solidify. The gel is covered with 1 to 2 mm cold electrophoresis buffer and the comb is carefully removed. Prepared samples containing the proteins of interest are diluted with sample buffer and loaded into wells. Electrophoresis is carried out for 3 to 6 hours at 4°C. The gel is placed into a vertical tank transfer

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Current Protocols in Cell Biology (2002) 6.7.1-6.7.13

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apparatus (*UNIT 6.2*), and the proteins are transferred overnight onto an immobilization matrix (PVDF membrane). The membrane is blocked and probed with a polyclonal primary antibody specific for the antigen of interest and, after washing, a conjugated secondary antibody is introduced. After incubation the membrane is washed again, and the protein bands of interest are illuminated with a chemiluminescent detection system.

Materials

SeaKem HGT(P) agarose (Bio Whittaker or equivalent)
1× electrophoresis buffer, 4°C (see recipe)
Protein samples
2× sample buffer (see recipe)
0.25× transfer buffer without methanol (see recipe)
Blocking buffer (*UNIT 6.2*) containing 5% (w/v) nonfat dry milk, fresh
Antibodies:
  Primary: rabbit anti-vWF (Dako)
  Secondary: donkey horseradish peroxidase–linked anti-rabbit Ig (Amersham Pharmacia)
ECL Western Blotting Analysis system (Amersham Pharmacia)

Aluminum foil
Boiling waterbath (optional)
Horizon 20-25 horizontal electrophoresis apparatus (Life Technologies) or equivalent
Teflon comb (e.g., 1 × 9–mm, 20-well)
Pipet with fine tip or equivalent
0.45-µm Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore)
Additional reagents and equipment for protein transfer to membranes and immunoblotting (*UNIT 6.2*)

Cast agarose gels

1. Weigh 1.2 g SeaKem HGT(P) agarose and transfer to a 250-ml flask containing 200 ml of 1× electrophoresis buffer. Add a Teflon-coated magnetic stir bar and tightly cover the flask with aluminum foil.

2. Heat in a boiling water bath, with slow mixing to avoid bubbles, until clear. Alternatively, microwave until boiling

   CAUTION: *Whenever a solution is heated in a microwave the chance of superheating ("boil up") is always present. Protective gloves, gown, and eyewear should be worn at all times.*

3. Assemble Horizon 20-25 horizontal electrophoresis apparatus or equivalent according to manufacturer's recommendations.

   The wedge shaped casting dams of the Horizon (Life Technologies) horizontal apparatus are easily placed in backwards, resulting in leaking of liquid agarose. Care should be exercised so that the dams form a perpendicular angle with the UVT tray.

4. Cool agarose to 55° to 60°C and pour the molten agarose into the electrophoresis apparatus to a depth of 4 mm.

   It is often helpful to prewarm the electrophoresis apparatus with warm water or in an oven to avoid cooling the agarose and causing the agarose to solidify unevenly.

5. Carefully insert the desired (e.g., 1 × 9–mm, 20-well) Teflon comb with care to avoid bubbles.

Removing the comb while the agarose is still in a molten state and then reinserting often eliminates the formation of bubbles under the teeth of the comb.

6. After the agarose has solidified, place the apparatus at 4°C and allow the agarose to age 20 to 30 minutes.

   The electrophoresis is to be carried out at 4°C. The apparatus may either be placed in a cold room, the electrophoresis buffer can be circulated through a refrigeration unit, or the apparatus can be packed in wet ice.

7. Overlay the solidified gel with 2 to 3 mm electrophoresis buffer, 4° C.

8. Remove the sample comb by lifting vertically in one smooth motion.

   It is helpful to hold the gel down with the gloved fingers of the other hand to keep the gel from being pulled up when the comb is removed.

Prepare and load sample

9. Dilute the protein samples to twice the desired concentration using distilled water. Immediately add the diluted sample to an equal volume of 2× sample buffer.

10. Load a sample volume of 10 to 15 µl into the bottom of each sample well using a pipet with a fine tip or equivalent.

    Prior to sample loading examine each sample well to ascertain that air bubbles are not trapped in the well.

Electrophorese gel (also see UNIT 6.1)

11. Run samples into the gel matrix at a constant current of 25 mA for ~30 min or until the sample dye has completely entered the gel.

12. Pause the electrophoresis and decrease the level of the electrophoresis buffer to ~1 mm above the upper surface of the gel. Increase the constant current to 50 mA and run for an additional 3 to 4 hr, or until the marker dye has migrated at least 6 to 7 cm.

Blotting the gel

13. Perform immunoblotting to a 0.45-µm Immobilon-P polyvinylidene fluoride (PVDF) membrane in a tank transfer system as described (*UNIT 6.2*), with the exception of the following conditions:

    a. Electrophoretically transfer at a constant current of 100 mA at 4°C overnight.
    b. Use 0.25× transfer buffer without methanol.
    c. Use blocking buffer containing 5% (w/v) nonfat dry milk.

Immunodetect protein

14. Perform immunoprobing with directly conjugated secondary antibody as described (*UNIT 6.2*), except with the following variations which are specific for immunodetection of von Willebrand Factor protein:

    a. Dilute primary antibody, rabbit anti-vWF, to a concentration of 1:4000.
    b. Dilute secondary antibody, donkey horseradish peroxidase–linked anti-rabbit Ig, to a concentration of 1:2000.

Visualization

15. Visualize von Willebrand factor protein on the PVDF membrane by meticulously following the recommendations enclosed in the ECL Western Blotting Analysis system.

Visualization with Luminescent Substrates is discussed in UNIT 6.2. To determine the optimal concentration of antibody, run a preliminary gel followed by immunoblotting. Cut the blot into several vertical test strips, each containing 1 to 2 lanes. Use the supplier's recommended concentration of antibody as a starting point and process the test strips with 3- to 10- to 30-fold increased and decreased antibody concentrations in separate containers.

ALTERNATE PROTOCOL

AGARAOSE GEL ELECTROPHORESIS WITH IN-GEL ANTIBODY ANALYSIS

This alternative protocol describes a method for separating large plasma proteins using SDS agarose electrophoresis and visualizing the protein of interest directly in the gel with a ^{125}I-radiolabeled rabbit antibody, in this case, to human von Willebrand factor protein, and autoradiography (see also *UNIT 6.3*). The methodology applied to agarose electrophoresis of von Willebrand factor is outlined below. The gel preparation consists of mating two glass plates on a horizontal surface separated by a 0.5-mm spacer. A sheet of GelBond support film is fixed to the glass plate to provide support for the agarose. Agarose at a concentration of 1.35% (w/v) is poured between the glass plate and the spacer plate. After the gel is solidified, the apparatus is disassembled and 1.0 × 0.1–cm wells are punched into the gel. The gel is placed into a horizontal electrophoresis chamber, presoaked wicks are attached to the gel, and sample volumes of 8 µl are loaded. The prepared samples are electrophoresed until the sample dye has migrated 8 to 10 cm. The gel is immediately fixed with isopropanol/glacial acetic acid fixing solution. After the gel is washed, it is blocked with ethanolamine/BSA. The gel, after a second series of washes, is incubated with ^{125}I-labeled anti-vWF antibody for 10 to 24 hours at room temperature. After extensive washing and drying the gel is placed in a cassette with film and exposed 1 to 5 days.

Materials

In-gel sample buffer, fresh (see recipe)
Borate saline buffer (BSB; see recipe)
Sample
0.5% (w/v) bromphenol blue in H_2O
Isopropanol
Agarose gel buffer (see recipe)
SeaKem HGT(P) agarose (FMC/BioWhittaker Molecular)
Agarose running buffer (see recipe)
Fixing buffer (see recipe)
Blocking buffer, in-gel (see recipe)
^{125}I-labeled rabbit anti-human vWF polyclonal antibody (Dako #A0082): radiolabel using protocol of choice and immunopurify (Hoyer and Shainoff, 1980; also see *UNIT 7.10*)
2% (w/v) human IgG (see recipe)
High-salt wash buffer (see recipe)

12 × 75–mm polypropylene tube
12.5 × 26.0 × 0.3–cm glass plate (Amersham Pharamacia Biotech)
12.5 × 24.0–cm spacer plate with adherent 0.5-mm spacers (Amersham Pharmacia Biotech)
12.4 × 25.8–cm GelBond film (Amersham Pharmacia Biotech)
Flexiclamps (Amersham Pharmacia Biotech)
20-ml syringe
60°C oven
Aluminum foil
Gelman Delux electrophoresis chamber (Gelman Sciences) or equivalent

104 × 253–mm paper electrophoresis electrode wicks (Amersham Pharmacia Biotech)
Flattened no. 2 cork borer
Forceps, fine
Horizontal rotary mixer
Forced hot-air dryer (optional)
Kodak X-Omatic film cassette with Lanex screens and film

CAUTION: When working with radioactivity, take apptopriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area, following the guidelines provided by your local radiations safety officer (also see *APPENDIX 1D*).

Prepare sample

1. Prepare 3 ml fresh in-gel sample buffer.
2. Label a 12 × 75–mm polypropylene tube for each sample to be assayed. Add 20 µl borate saline buffer (BSB), 120 µl sample buffer, and 10 µl sample into each tube.
3. Cover each tube, vortex gently, and incubate 2 hr at 37°C.
4. Add 8 µl of 0.5% bromphenol blue to each sample and mix gently.

Prepare agarose gel

5. Prepare a boiling water bath by placing approximately 50 ml distilled water in a 250-ml beaker and incubating on a hot plate with magnetic stirring capabilities.
6. Use isopropanol to clean the 12.5 × 26.0 × 0.3–cm glass plate and 12.5 × 24.0–cm spacer plate with its adherent 0.5–mm spacers. Dry with a lint-free tissue (e.g., Kimwipe).

   It is advisable, because of the fragile nature of this gel, to prepare sufficient materials to pour a gel in reserve in the eventuality that one is rendered unusable.

7. Place a few milliliters of distilled water on the glass plate and adhere the hydrophilic side of a 12.4 × 25.8–cm GelBond film. Express any trapped air or excess water with a lint-free tissue.
8. Mate the glass plate with the adherent GelBond film and the spacer plate. Clamp with two flexiclamps.

   The gel-forming sandwich should consist of the glass plate (the larger of the two plates) with an adherent piece of GelBond film (hydrophilic side to the glass plate) and the spacer plate with its adherent spacer bars placed on top of the GelBond (Fig. 6.7.1).

9. Place the clamped plates and a 20-ml syringe in a 60°C oven for ~10 minutes to equilibrate.
10. Measure 40 ml agarose gel buffer into a 50-ml Erlenmeyer flask and add a Teflon-coated magnetic stir bar. Pour 0.54 g SeaKem HGT(P) agarose into the flask, tightly cover with aluminum foil, and place the flask into the boiling water bath (step 5). Dissolve the agarose with constant stirring. After the solution becomes clear, continue boiling an additional 10 min.
11. Remove the heated glass plate assembly (gel-forming sandwich) and the 20-ml syringe from the oven. Quickly fill the heated syringe with 20 ml hot agarose, and holding the plate assembly at an ~75° angle, fill the narrow space in the plate assembly using a back and forth motion to prevent bubbles from being trapped in the gel.

   Use of a needle with the syringe is optional.

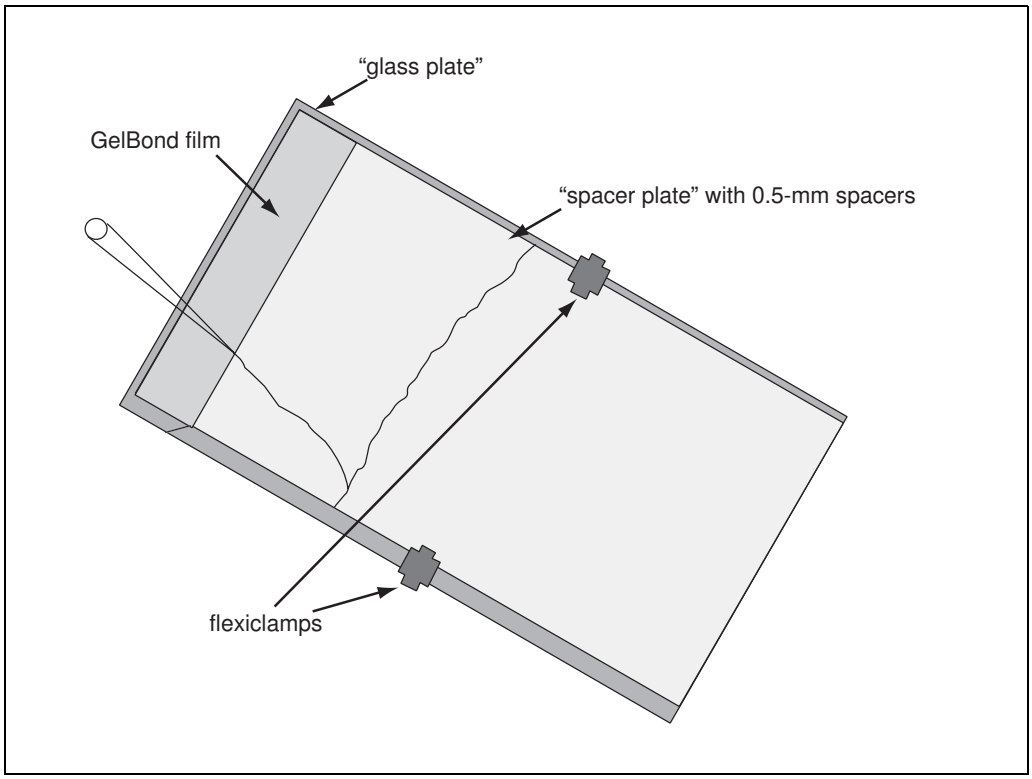

Figure 6.7.1 Diagram of casting gel in the Alternate Protocol. The layers include (back to front): the "glass plate" (the larger of the two plates), GelBond film (hydrophilic side adherent to the glass plate), and the "spacer plate" with attached 0.5-mm spacer bars. The agarose fills the narrow space between the GelBond and the "spacer plate."

12. After filling, lay the plate assembly flat and allow to cool and solidify ~45 min.

Electrophorese gel

13. Position the Gelman Delux electrophoresis chamber or equivalent electrophoresis apparatus on a flat surface and fill each electrode chamber with 400 to 450 ml agarose running buffer. Place 104 × 253–mm paper electrophoresis electrode wicks in each chamber to equilibrate in running buffer.

14. Remove the flexiclamps. Insert a thin spatula blade between the spacer plate and the gel attached to the glass plate. Carefully pry upward to separate the spacer plate. Examine the gel against a bright light for bubbles, thin areas, or areas of separation from the GelBond film.

    The agarose gel should now be attached to the GelBond support backing and be easily handled.

15. Punch the required number of 0.1 × 10-mm wells using a flattened no. 2 cork borer. Use a fine set of forceps to remove the agarose from the interior of each well.

    It is helpful to construct a template to place under the gel to facilitate punching the wells in an evenly spaced straight line. Also, it is necessary to work quickly because these gels are very thin and dry out rapidly.

16. Place a glass plate across the bridge on the electrophoresis chamber and place the gel attached to the GelBond on top of the plate. Affix the presoaked wicks (step 13) on each side of the gel so that there is continuity between the agarose running buffer in the electrode chambers and the gel.

17. Load 8 µl of sample into each well. Place the cover over the electrophoresis apparatus and connect the power supply.

18. Electrophorese at 25 V for ~30 min at room temperature to allow the samples to enter the gel matrix. Increase the power supply to 50 V and continue electrophoresis until the dye marker has migrated 8 to 10 cm from the wells (~3 to 5 hr).

Fix gel

21. Add 200 ml fixing buffer to a container appropriate to the size of the gel. Carefully remove the paper wicks and place the gel in fixing buffer 1 hr without agitation.

    The gel can also fix overnight in fixing buffer for a convenient stopping point.

22. Wash the gel 1 hr with 200 to 300 ml BSB with gentle mixing on a horizontal rotary mixer.

    A prerinse ~1 to 2 min before the wash with ~100 ml BSB is recommended

23. Prepare 250 ml fresh in-gel blocking buffer. Block gel 1 hr with gentle mixing.

24. Prerinse gel with ~100 ml BSB. Wash 1 hr with 200 to 300 ml BSB.

Immunodetect protein

25. Prepare a solution of dilute ^{125}I-labeled rabbit anti-human vWF polyclonal antibody in BSB to a concentration of ~2×10^6 cpm in a volume sufficient to cover the gel. Add 0.5 ml of 2% human IgG to the antibody solution.

    The majority of the gels can be covered with 50 to 70 ml.

    In an effort to minimize the volume of radioactive solutions, the authors' laboratory keeps a supply of dedicated plasticware to accommodate a wide variety of gel sizes. Strict laboratory precaution should be exercised in the preparation, handling, and disposal of radioactive materials.

26. Incubate the gel in the antibody solution at least 16 to 24 hr with gentle mixing.

27. Discard the antibody solution, adhering to standard radiation safety waste disposal protocols.

28. Wash the gel 1 hr in 200 ml high-salt wash buffer with gentle mixing using a horizontal rotary mixer.

29. Repeat the wash an additional three to four times.

    The washes should be monitored and continued until all excess ^{125}I is removed.

30. Wash 1 hr in 200 ml distilled water. Repeat once.

31. Dry the gel with a forced hot-air dryer directed on the gel, or air dry the gel.

Autoradiograph the gel

32. Place the gel in a Kodak X-Omatic film cassette with a Lanex screen. Under dark room conditions, place a piece of Kodak X-Omat film on the gel and then incubate the cassette at −70°C. Expose for an appropriate amount of time.

    Times vary from 1 to 5 days. It is helpful to secure the gel to the cassette with tape to keep it from changing position.

REAGENTS AND SOLUTIONS

*Use deionized or distilled water in all recipes and protocol steps. For common solutions, see **APPENDIX 2A**; for suppliers, see **SUPPLIERS APPENDIX**.*

Agarose gel buffer

0.05 M Na_2HP0_4
0.1% (w/v) SDS
Adjust pH to 7.0 with concentrated HCl
Filter and store up to 3 months at room temperature

Agarose running buffer

0.1 M Na_2HP0_4
0.1% (w/v) SDS
Adjust pH to 7.0 with concentrated HCl
Store up to 3 months at room temperature

Blocking buffer, in-gel

15 ml 16.6 M ethanolamine (1.0 M)
250 mg fatty-acid-free Fraction V BSA
Add H_2O to ~200 ml
Adjust pH to 8.0 with concentrated HCl
Add H_2O to 250 ml
Prepare fresh on the day of use

Borate saline buffer (BSB)

15.4 g boric acid (36 mM)
65.06 g NaCl (143 mM)
1.40 g NaOH (0.005 N)
Add 1.56 ml concentrated HCl to adjust pH to 7.83
Adjust volume to 7 liters with H_2O
Store up to 3 months at room temperature

Electrophoresis buffer, 1×

Dilute 10× TAE (see recipe) 1/10 in water. Add 10 ml of 20% (w/v) SDS (*APPENDIX 2A*) per 2 liters. Final concentrations are 40 mM Tris-acetate, 1 mM EDTA, and 0.1% (w/v) SDS. Final pH is 7.8 to 8.3. Store up to 1 week at room temperature.

The solution is also referred to as 1× TAE-SDS. Two liters are required for the application.

Fixing buffer

50 ml isopropanol (25%)
20 ml glacial acetic acid (10%)
130 ml H_2O
Prepare fresh on the day of use

High-salt wash buffer

56.78 g Na_2HPO_4 (0.1 M final)
233.6 g NaCl (1 M final)
Adjust pH to 7.0 with concentrated HCl
Add H_2O to 4 liters
Store up to 3 months at room temperature

Human IgG, 2%

2.0 g human IgG in 100 ml BSB (see recipe)
Store in aliquots up to 1 year at −20°C

In-gel sample buffer

Stock solution
0.01M Na_2HP0_4
Adjust pH to 7.0 with HCl
Filter and store up to 3 months at room temperature
Working solution
To 3 ml stock solution add:
20.64 mg iodoacetamide
37.5 mg SDS
Prepare fresh

Sample buffer, 2×

20 ml 10× TAE (see recipe)
1 ml 20% (w/v) SDS (*APPENDIX 2A*)
20 ml glycerol
0.2 g bromphenol blue
Add H_2O to 200 ml
Store up to 1 year at room temperature

TAE, 10×

48.4 g Tris base (400 mM)
20 ml 0.5M EDTA (10 mM; *APPENDIX 2A*)
Adjust pH to 7.8 with glacial acetic acid
Adjust volume to 1 liter
Store up to 1 year at room temperature

Transfer buffer without methanol, 0.25× and 10×

For a 10× solution
250 mM Tris·Cl, pH 8.3 (*APPENDIX 2A*)
1.92 M glycine
1.0% (w/v) SDS
Store up to 1 year at room temperature
This is the stock solution for transfer buffer and is also known as Tris/glycine/SDS (TG/SDS).

For a 0.25× solution
Dilute 10× transfer buffer 1/40 in distilled water. Final concentrations are 6.25 mM Tris·Cl, 48 mM glycine, and 0.025% (w/v) SDS. Final pH is 8.3. Store up to 1 week at room temperature.
Four liters are required for the application.

Table 6.7.1 Troubleshooting Guide for Agarose Electrophoresis and Immunoblotting

Problem	Possible cause	Solution
Agarose electrophoresis		
Run time too long or too short	Buffer concentration too high or too low	Verify buffer preparation
	Voltage too high or too low	Review and increase or decrease voltage or current settings
Band spreads into other lanes	Sample diffusing out of well or into surrounding gel	Minimize time for sample loading and start electrophoresis promptly
Samples leak underneath gel	Bottom of well torn when removing comb	Remove comb slowly
Bands migrate at different rates (visualized as "smiles," "frowns," and "sneers")	Gel cast unevenly	Use level to verify that the apparatus is level
	Uneven heat distribution	Decrease power settings. Cool buffer to 4°C. Circulate buffer.
Bands not in straight lines (visualized as a "wiggle")	Artifacts in wells	Flush wells with electrophoresis buffer
		Inspect wells for trapped air bubbles
Bromphenol turns yellow	pH change during electrophoresis	Verify pH of buffer
		Circulate buffer during run
Immunoblotting		
High background	Insufficient blocking	Increase concentration and/or time of blocking step
	Overdevelopment	Remove membrane from substrate after 1 min
	Protein contamination	Wash or replace fiber pads and clean apparatus
	Incomplete washing	Increase wash time and volume
		Review recipe, especially Tween 20
	Primary or secondary antibody too concentrated	Review supplier's recommendations
		Run test strips to optimize reactions
Weak signal or no reaction	Sample load insufficient	Increase amount of sample
	Low antibody specificity	Increase antibody concentration
	Antigen not transferred	Increase transfer time. Stain membrane for protein transfer.
	Conjugate not active	Mix more thoroughly

COMMENTARY

Background Information

The major use of agarose in protein analysis is its application as a matrix for molecular sieving; however, it is also widely used as a material that can be modified to form an affinity matrix for affinity chromatography. Because of its ability to separate proteins of very large size, it is also utilized for electrophoresis and preparation of very large proteins, ranging from several million to approximately fifty-thousand daltons. Agarose has the advantage of being nontoxic. In addition, it may be melted to allow recovery and further studies of the separated protein, and an excised band may be directly injected into an animal for immunization. Also, the use of native gels allows separation and recovery of nondenatured proteins for functional studies.

The electrophoresis procedure outlined above illustrates the ability of agarose to separate proteins of molecular weights that exceed

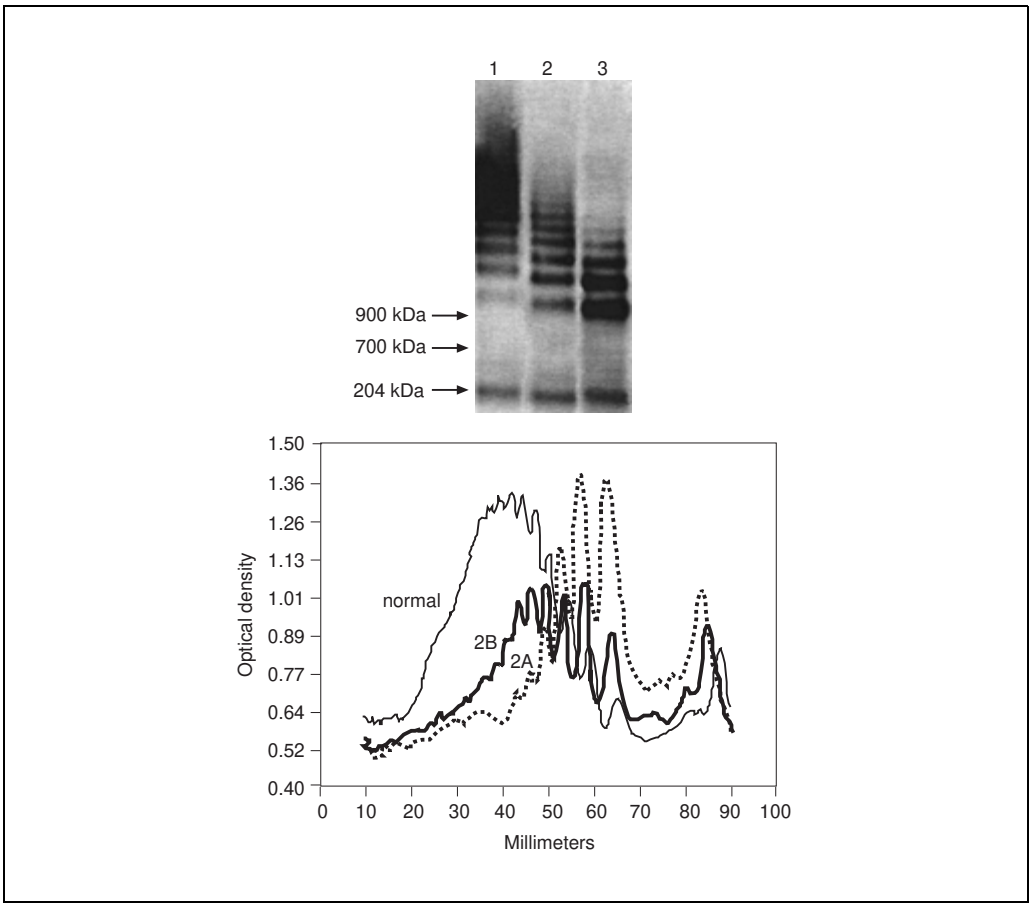

Figure 6.7.2 Luminograph of vWF multimers from normal plasma (lane 1), von Willebrand disease Type 2B plasma (lane 2), and von Willebrand disease Type 2A plasma (lane 3). A densitometric tracing is seen below. Reproduced with permission from Krizek and Rick (2000).

1×10^6 Da. For smaller proteins, higher concentrations of agarose (e.g., 3%) and shorter blotting times may be used. In instances where electroblotting cannot be carried out because of precipitation of the proteins during transfer due to separation from detergent, the proteins can be immobilized in the gel to prevent diffusion before immuno-identification. Immobilization of the proteins also allows for the use of sequential antibodies for identification of protein bands. Additionally, in-gel identification may also be important if there is uneven transfer of proteins due to dissimilar transfer characteristics. Immobilization of the proteins in the gel is accomplished with the use of an agarose gel that is modified by the addition of glycidol to yield a glyceryl agarose that contains aldehyde groups after oxidation by periodate. Proteins are covalently bound in the gel after electrophoresis by reaction of their amino groups with the aldehydes in the presence of the reducing agent, sodium cyanoborohydride. Further direct probing with antibodies can be carried out without the need for transfer to another support (Shainoff, 1993). Composite gels of agarose or glyoxal agarose have also been prepared to provide differing degrees of sieving (Peacock and Dingman, 1968; Shainoff, 1993).

Although few proteins are as large as vWF, the evaluation of von Willebrand factor multimers illustrates an important clinical application of the use of agarose as a medium for electrophoresis. Assessment for the presence of the largest multimers is physiologically important for the diagnosis of von Willebrand disease and for selection of the most appropriate treatment (Rick, 2001). The original procedure for this analysis (see Alternate Protocol, with minor modifications) included glycidol in the agarose which served to aid in the fixation of the protein bands while the further washing and antibody identification steps were accomplished (Hoyer and Shainoff, 1980). It was subsequently found that diffusion of protein bands was not a limiting factor with vWF, and this immobilization step was eliminated from the method.

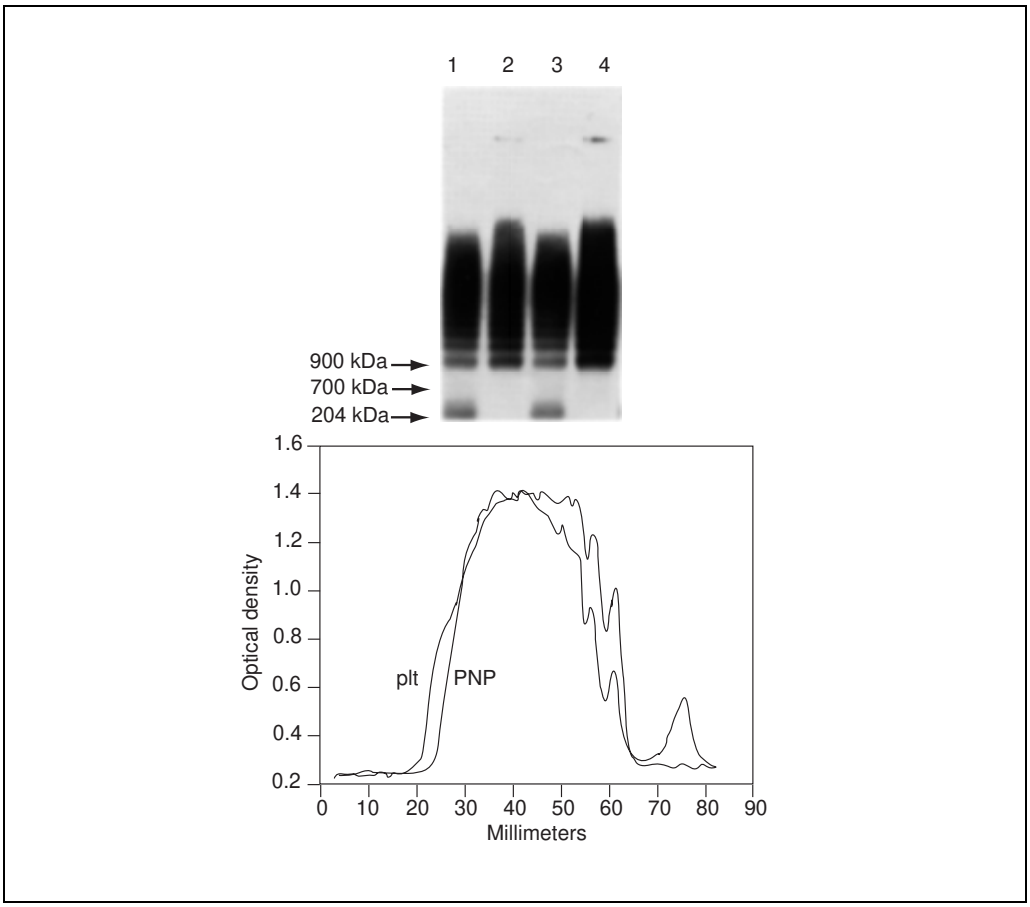

Figure 6.7.3 Luminograph of vWF multimers in pooled normal plasma (PNP; lanes 1 and 3) and vWF multimers extracted from platelets (plt; newly synthesized vWF; lanes 2 and 4). The densitometric tracing below shows the earlier "take-off" of the platelet vWF, indicating larger multimers. Adapted with permission from Krizek and Rick (2000).

Analysis of the distribution of von Willebrand factor multimers is also used to assess the function of an important protease that cleaves von Willebrand factor and decreases the prothrombotic "unusually high-molecular-weight" multimers of von Willebrand factor; these multimers are initially synthesized and secreted into the circulation, but are cleaved by the vWF protease (Krizek and Rick, 2001; Aronson, Krizek, and Rick, 2001).

Critical Parameters and Troubleshooting

It is important to maintain the temperature at 4°C during electrophoresis using the horizontal bed (see Basic Protocol). The blotting step must be carried out for a sufficient time to allow transfer of very high-molecular-weight proteins. Thorough washing after blocking buffer and antibody additions is important in both protocols. See Table 6.7.1 for troubleshooting agarose gel electrophoresis and immunoblotting.

Anticipated Results

The radiographs that result from the chemiluminescent and radioactive detection procedures show a wide distribution of multimer sizes of normal von Willebrand factor. In certain subtypes of von Willebrand disease (i.e., Type 2) there is a marked or modest decrease in the higher-molecular-weight multimers (Fig. 6.7.2). If newly synthesized von Willebrand factor is extracted from platelets, the unusually high-molecular-weight multimers are seen (Fig. 6.7.3). In samples that are incubated under conditions that activate the von Willebrand factor protease, a decrease in the high and intermediate sized multimers is seen (Fig. 6.7.4; Rick and Krizek, unpub. observ.).

Time Considerations

Both protocols should be started in the morning to allow sufficient time for electrophoresis. Horizontal electrophoresis and blotting can be completed within 48 hours: blotting is conveniently completed overnight and detec-

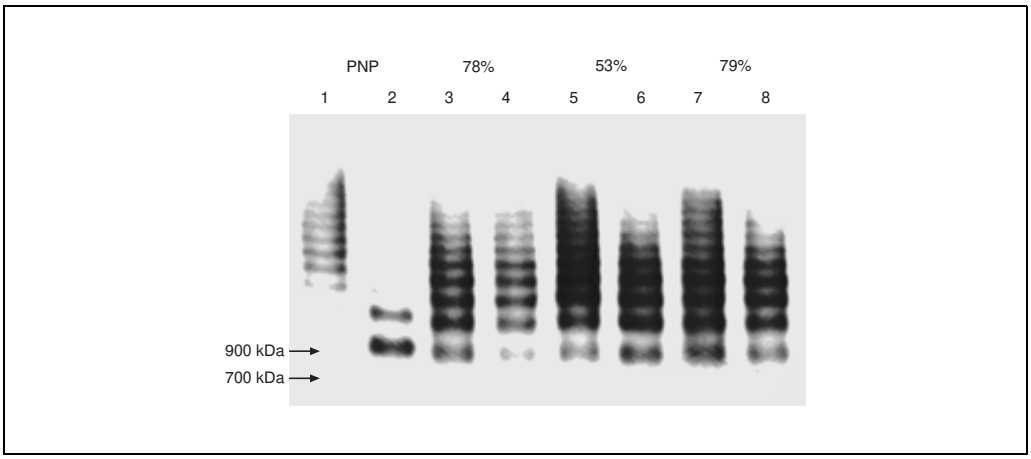

Figure 6.7.4 Luminograph of pooled normal plasma (PNP) showing normal vWF multimers (lane 1) and proteolysed multimers of normal vWF from PNP after exposure to conditions that activate the vWF protease (lane 2). Paired samples from patients with thrombotic thrombocytopenic purpura who have an inhibitor to the vWF protease are seen in lanes 3 to 8. Odd lanes contain plasma samples using conditions that do not activate the vWF protease, and even lanes contain the paired sample that was exposed to conditions that activate the protease. Very little proteolysis is observed in these patients' plasmas due to the presence of an inhibitor (even lanes). The numbers above the patient lanes indicate the retention of the high-molecular-weight multimers.

tion procedures can be completed the next day. The Alternate Protocol takes up to 5 or more days to obtain results, largely due to the time required for the incubation with antibody and development of the autoradiogram. Also, a radiolabeled antibody specific for the protein to be identified must be available.

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Key References

Krizek and Rick, 2000. See above.

This original paper of the procedure and use of immunoblotting and chemiluminescence for ararose gel electrophoresis provides the background and reasons for the development of this assay in the clinical laboratory setting.

Hoyer and Shainoff, 1980. See above.

This paper outlines the original "in-gel" procedure for the electrophoresis of very high molecular weight proteins and provides examples of its usefulness in understanding the structure of von Willebrand factor.

Shainoff, 1993. See above.

This reference provides general background and the rationale for the use of modified ararose for the separation and identification of (large) proteins by electrophoresis.

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Fluorescence Detection of Glycoproteins in Gels and on Electroblots

The co-translational and post-translational covalent attachment of oligosaccharides to proteins is a common cellular event in eukaryotes, regulated by a variety of glycosidases and glycosyltransferases (Beeley, 1985; Reuter and Gabius, 1999; *UNIT 15.2*). Glycosylation profiles are dynamic, changing during development, differentiation, and disease. Glycosylation of proteins is critical to the adhesiveness of microorganisms and cells, cellular growth control, cell migration, tissue differentiation, and inflammatory reactions. Differences in glycosylation profiles are often used as a "barometer" to assess disease states. With the advent of proteomics, genome-wide protein analysis, there is renewed interest in the rapid and sensitive identification of glycoproteins by methods that do not require degradation of the protein component of the macromolecule (Packer et al., 1999; Hirabayashi et al., 2001). Until recently, there have been relatively few methods available for the direct analysis of glycans on proteins transferred to membranes and most especially of glycans on proteins within polyacrylamide gels (Packer et al., 1999; Koketsu and Linhardt, 2000; Raju, 2000) . Such methods could readily be incorporated into integrated proteomics platforms that utilize automated gel stainers, image analysis workstations, robotic spot excision instruments, protein digestion work stations, and mass spectrometers (Patton, 2000a,b).

There are two principal approaches to the detection of glycoproteins in gels and on blots; reacting carbohydrate groups by periodate/Schiff's base (PAS) chemistry and noncovalent binding of specific carbohydrate epitopes using lectin-based detection systems. The PAS method involves oxidation of carbohydrate groups, followed by conjugation with a chromogenic substrate (acid fuchsin, Alcian Blue), a fluorescent substrate (dansyl hydrazine, 8-aminonaphthalene-1, 3,6-trisulfonate, Pro-Q Emerald dye), biotin hydrazide, or digoxigenin hydrazide. Signal is detected directly in the case of the chromogenic or fluorescent conjugates and indirectly using enzyme conjugates of antibodies for bound digoxigenin or enzyme conjugates of streptavidin for bound biotin. Lectins permit detection of certain structural subclasses of glycoproteins by similar methods to those used in standard immunoblotting applications. Typically, lectin conjugates of biotin along with enzyme conjugates of streptavidin or direct conjugates of lectin and enzyme are utilized along with chromogenic, fluorogenic, or chemiluminescent substrates. Just as in immunoblotting, the most popular enzymes used to detect lectin or streptavidin are alkaline phosphatase and horseradish peroxidase.

This unit describes periodate/Schiff's base and lectin methods for the detection of glycoproteins. The Pro-Q Emerald 300 glycoprotein detection method permits fluorescent direct detection of glycoproteins in gels (see Basic Protocol 1) or on blots (see Alternate Protocol) without the use of enzyme amplification systems. The method may also be used to detect lipopolysaccharides, constituents of the outer membrane surrounding gram-negative bacteria. The Pro-Q glycoprotein blot stain protocol for concanavalin A (see Basic Protocol 2) is suitable for the detection of glycoproteins containing α-mannopyranosyl and α-glucopyranosyl residues on blots using an alkaline phosphatase–based signal amplification system. Using different enzyme-lectin conjugates, such as alkaline phosphatase conjugates of wheat germ agglutinin or *Griffonia simpliifolia* lectin II (GS-II), the method can be adapted to the detection of other glycan structures present in glycoproteins.

Contributed by Wayne F. Patton

Current Protocols in Cell Biology (2002) 6.8.1-6.8.15

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BASIC PROTOCOL 1

FLUORESCENT DETECTION OF GLYCOPROTEINS IN POLYACRYLAMIDE GELS

Pro-Q Emerald 300 Glycoprotein Gel Stain Kit provides a robust method for differentially staining glycosylated and non-glycosylated proteins in the same gel. The technique combines the green fluorescent Pro-Q Emerald 300 glycoprotein stain with the orange-red fluorescent SYPRO Ruby total protein gel stain.

Pro-Q Emerald 300 glycoprotein stain reacts with periodate-oxidized carbohydrate groups, creating a bright green-fluorescent signal on glycoproteins. Using this stain allows detection of <1 ng glycoprotein/band, depending upon the nature and the degree of glycosylation, making it 100-fold more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye (rosaniline). The green-fluorescent signal from the Pro-Q Emerald 300 stain can be visualized using a standard 300-nm UV (UV-B) illumination source. The Pro-Q Emerald 488 Glycoprotein Gel Stain Kit is quite similar to the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, but it is optimized for use with gel scanners equipped with 470- to 488-nm lasers. The Pro-Q Emerald dye staining method is more reliable than mobility-shift assays using glycosidases since even glycoproteins that are not susceptible to deglycosylation with specific enzymes may readily be identified as glycoproteins.

After detecting glycoproteins with Pro-Q Emerald 300 dye, total protein profiles may be detected using SYPRO Ruby protein gel stain. SYPRO Ruby protein gel stain interacts noncovalently with basic amino acid residues in proteins. The stain is capable of detecting <1 ng of protein/band, making it at least as sensitive as the best silver staining procedures available. The orange-red fluorescent signal from SYPRO Ruby protein gel stain can be visualized using a standard 300-nm UV (UV-B) illumination source, or alternatively may be excited using 470- to 488-nm laser, gas discharge, or xenon arc sources.

Materials

Protein sample of interest
Fix solution (see recipe)
Wash solution (see recipe)
Pro-Q Emerald 300 Glycoprotein Gel Stain Kit (Molecular Probes) containing:
- 50× Pro-Q Emerald 300 reagent, concentrate in DMF
- Pro-Q Emerald 300 dilution buffer
- Periodic acid (oxidizing reagent; see recipe)
- CandyCane glycoprotein molecular weight standards (see recipe), sufficient volume for approximately 20 gel lanes
- SYPRO Ruby protein gel stain

Deionized, high quality water
10% (v/v) methanol or ethanol, spectroscopy grade (optional)
7% (v/v) glacial acetic acid (optional)

Polystyrene staining dishes (e.g., a weighing boat for minigels or larger container for larger gels)
Orbital shaker
UV transilluminator
Photographic camera or CCD camera and appropriate filters

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*)

Run gel

1. Prepare the protein samples of interest (e.g., crude protein isolates, cell lysates, serum, partially purified plasma membranes) for SDS-polyacrylamide gel electrophoresis.

   Typically, the protein sample is diluted to ~10 to 100 µg/ml with 2× sample buffer, heated for 4 to 5 min to 95°C, and 5 to 10 µl of diluted sample is then applied per gel lane for 8 × 10–cm gels. Larger gels require proportionally more material.

   For convenience, CandyCane glycoprotein molecular weight standards may also be applied to a lane or two. Typically, 2 µl of this standard is diluted in 6 µl of sample buffer and heated in the same manner as the samples to be characterized. These standards contain a mixture of glycosylated and non-glycosylated proteins ranging from 14 to 180 kDa in molecular weight. The standards serve as molecular weight markers and provide alternating bands as positive and negative controls for glycoprotein and total protein detection. Each protein is present at 0.5 mg/ml.

2. Separate proteins by SDS-polyacrylamide gel electrophoresis using standard methods (*UNIT 6.1*).

   The staining procedure is optimized for gels that are 0.5- to 1- mm thick.

Fix gel

3. After electrophoresis, fix the gel by immersing it in 75 to 100 ml of fix solution in a polystyrene staining dish and incubating with gentle agitation (e.g., on an orbital shaker at 50 rpm) for 45 min at room temperature.
4. Wash the gel by incubating it in 50 ml wash solution with gentle agitation for 10 min at room temperature. Repeat wash one additional time.
5. Oxidize the gel in 25 ml periodic acid solution with gentle agitation for 30 min at room temperature.
6. Wash the gel in 50 ml wash solution with gentle agitation for 5 to 10 min at room temperature. Repeat this washing step two additional times.

Stain gel for glycoproteins

7. Prepare fresh Pro-Q Emerald 300 staining solution by diluting the 50× Pro-Q Emerald 300 concentrate reagent 50-fold into Pro-Q Emerald 300 dilution buffer.

   For example, dilute 500 µl of 50× Pro-Q Emerald 300 reagent into 25 ml dilution buffer to make enough staining solution for one 8 × 10–cm gel.

8. Incubate the gel in subdued light in 25 ml of Pro-Q Emerald 300 staining solution (step 7) while gently agitating for 90 to 120 min.

   The signal can be seen after ~20 min and maximum sensitivity is reached at ~120 min. Staining overnight is not recommended.

9. Wash the gel with 50 ml wash solution for 15 min at room temperature. Repeat this wash one additional time. Do not leave the gel in wash solution for >2 hr, as the staining signal will start to decrease.
10. Visualize the stain using a standard UV transilluminator.

    The Pro-Q Emerald 300 stain has an excitation maximum at ~280 nm and an emission maximum near 530 nm. Stained glycoproteins can be visualized using a 300 nm UV-B transilluminator.

11. Document results before proceeding to the next step. Use a photographic camera or CCD camera and the appropriate filters to obtain the greatest sensitivity (see Fig. 6.8.1).

    Pro-Q Emerald dye signal will fade after SYPRO Ruby dye staining.

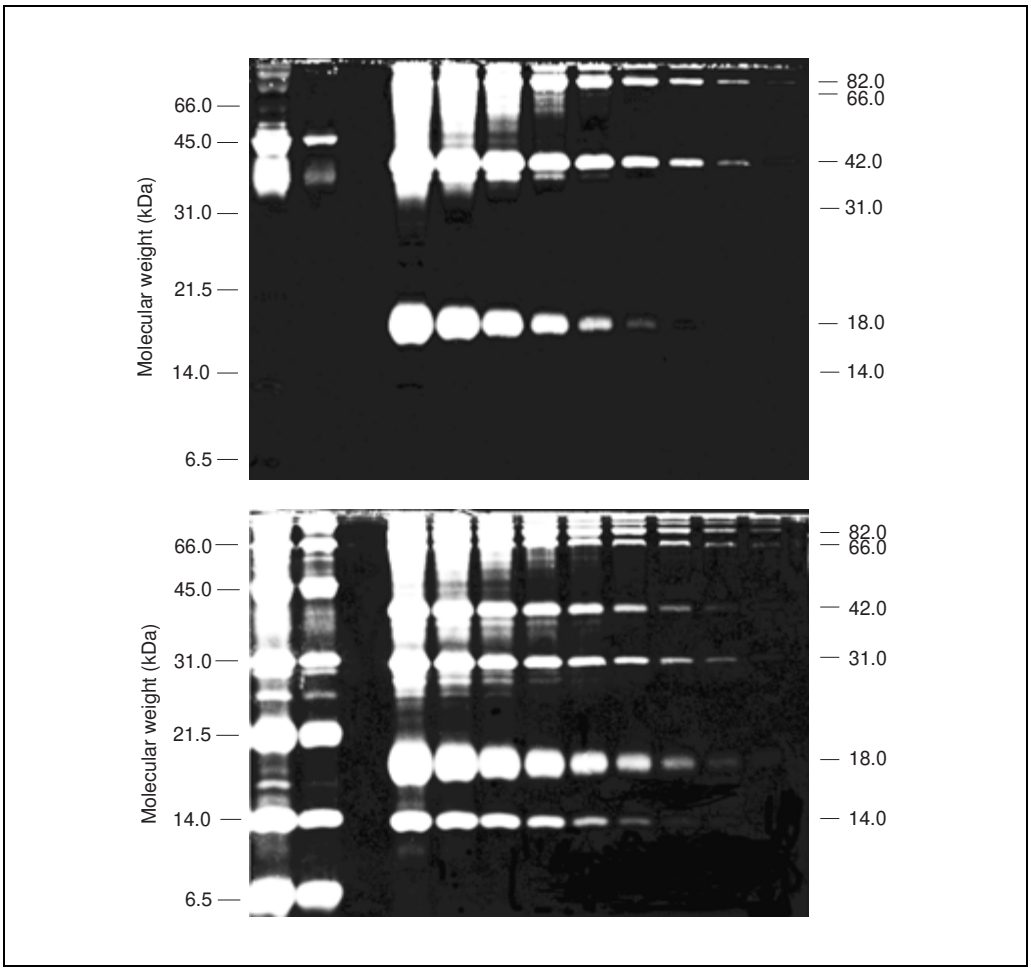

Figure 6.8.1 Sensitivity and specificity of glycoprotein detection in 13% SDS-polyacrylamide gels using Pro-Q Emerald 300 glycoprotein gel stain. (**A**) Detection of glycoproteins using Pro-Q Emerald 300 glycoprotein gel stain. (**B**) Detection of the total protein profile using SYPRO Ruby protein gel stain. Lanes 1 and 2, broad range molecular weight markers containing the 45-kDa glycoprotein ovalbumin, 1000 and 250 ng, respectively. Lane 3, blank. Lanes 4 to 12, CandyCane molecular-weight markers, a mixture of glycosylated and unglycosylated proteins (1000 to 3.9 ng, as two-fold serial dilutions). Gels were imaged using a Lumi-Imager F1 instrument (Roche Molecular Biochemicals). Both dyes were excited using the instrument's 300-nm UV-B transilluminator and images were captured using the instrument's cooled CCD camera. Pro-Q Emerald 300 dye signal was collected using the standard 520-nm band-pass emission filter. Gels were then stained with SYPRO Ruby protein gel stain and SYPRO Ruby dye signal was collected using the 600-nm band-pass emission filter provided with the instrument. Figure courtesy of Courtenay Hart, Molecular Probes.

Stain the gel for total protein

12. In order to counter-stain non-glycosylated proteins in the sample, pour the SYPRO Ruby protein gel staining solution into a small, clean plastic dish.

    For one or two standard-size mini-gels, use ~50 ml to 100 ml of staining solution; for larger gels, use 500 to 750 ml.

13. Place the gel into the staining solution and gently agitate (e.g., on an orbital shaker at 50 rpm) at room temperature.

    The staining time ranges from 90 min to 3 hr, depending upon the thickness and percentage of polyacrylamide in the gel. Specific staining can be seen in as little as 30 min. However, a minimum of 3 hr of staining is required for the maximum sensitivity and linearity. For convenience, gels may be left in the dye solution overnight or longer without over staining.

14. After staining, rinse the gel in water for 30 to 60 min to decrease background fluorescence.

    Alternatively, to further decrease background fluorescence, the gel can be washed in a mixture of 10% methanol (or ethanol) and 7% acetic acid for 30 min instead of water. The gel may be monitored periodically using UV illumination to determine the level of background fluorescence.

15. Visualize the stain using an appropriate method (see Fig. 6.8.1).

    The stained gel is best viewed on a standard 300-nm UV-B transilluminator, though stain will be visible using a 254-nm UV-C or 365-nm UV-A transilluminator. Gels may also be visualized using various laser scanners: 473-nm (SHG) laser, 488-nm argon-ion laser, or 532-nm (YAG) laser. Alternatively, use a xenon arc lamp, blue fluorescent light, or blue light–emitting diode (LED) source. Gels may be photographed by Polaroid or CCD camera. Use Polaroid 667 black-and-white print film and the SYPRO protein gel stain photographic filter (Molecular Probes).

FLUORESCENT DETECTION OF GLYCOPROTEINS ON ELECTROBLOT MEMBRANES

ALTERNATE PROTOCOL

Pro-Q Emerald 300 Glycoprotein Blot Stain Kit provides a robust method for differentially staining glycosylated and non-glycosylated proteins on the same electroblot. The technique combines the green fluorescent Pro-Q Emerald 300 glycoprotein stain with the orange-red fluorescent SYPRO Ruby total protein gel stain.

The Pro-Q Emerald 300 glycoprotein stain reacts with periodate-oxidized carbohydrate groups, creating a bright green-fluorescent signal on glycoproteins. Using this stain, allows detection of <1 ng glycoprotein/band, depending upon the nature and the degree of glycosylation, making it 100-fold more sensitive than the standard periodic acid–Schiff base method using acidic fuchsin dye (rosaniline). The green-fluorescent signal from Pro-Q Emerald 300 stain can be visualized using a standard 300-nm UV (UV-B) illumination source. The Pro-Q Emerald 488 Glycoprotein Blot Stain Kit is quite similar to the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, but is optimized for use with gel scanners equipped with 470- to 488-nm lasers. The staining method is more reliable than mobility-shift assays using glycosidases since even glycoproteins that are not susceptible to deglycosylation with specific enzymes may readily be identified as glycoproteins.

After detecting glycoproteins with Pro-Q Emerald 300 dye, total protein profiles may be detected using SYPRO Ruby protein blot stain. SYPRO Ruby protein blot stain interacts noncovalently with basic amino acid residues in proteins. The stain is capable of detecting <4 ng of protein/band, making it at least as sensitive as the best colloidal gold staining procedures available. The orange-red fluorescent signal from SYPRO Ruby protein blot stain can be visualized using a standard 300 nm UV (UV-B) illumination source or alternatively may be excited using 470- to 488-nm laser, gas discharge, or xenon arc sources.

Materials

Protein sample of interest
PVDF membrane
Fix solution (see recipe)
Wash solution (see recipe)
Pro-Q Emerald 300 Glycoprotein Blot Stain Kit (Molecular Probes) containing:
  50× Pro-Q Emerald 300 reagent, concentrate in DMF
  Pro-Q Emerald 300 dilution buffer
  Periodic acid (oxidizing solution; see recipe)

CandyCane glycoprotein molecular weight standards (see recipe), sufficient volume for ~20 gel lanes
SYPRO Ruby protein blot stain
Methanol, spectroscopy grade (optional)
Glacial acetic acid (optional)

95°C heat block
Polystyrene staining dishes (e.g., weighing boat for minigels or larger containers for larger gels)
Orbital shaker
UV epi-illuminator
Photographic camera or CCD camera and appropriate filters

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*) and electroblotting (*UNIT 6.2*)

Run gel

1. Prepare the protein samples of interest (e.g., crude protein isolates, cell lysates, serum, partially purified plasma membranes) for SDS-polyacrylamide gel electrophoresis.

   Typically, the protein sample is diluted to ~10 to100 µg/ml with 2× sample buffer, heated for 4 to 5 min at 95°C, and then 5 to 10 µl of diluted sample is applied per gel lane for 8 × 10–cm gels. Larger gels require proportionally more material.

   For convenience, CandyCane glycoprotein molecular weight standards may also be applied to a lane or two. Typically, 2 µl of this standard is diluted in 6 µl of sample buffer and heated in the same manner as the samples to be characterized. These standards contain a mixture of glycosylated and non-glycosylated proteins ranging from 14 to 180 kDa in molecular weight. The standards serve as molecular weight markers and as alternate bands of positive and negative controls for glycoprotein and total protein detection. Each protein is present at 0.5 mg/ml.

2. Separate proteins by SDS-polyacrylamide gel electrophoresis using standard methods (*UNIT 6.1*).

   The procedure is optimized for gels that are 0.5- to 1-mm thick.

Prepare blot

3. After electrophoresis, transfer the proteins to PVDF membrane using standard electroblotting procedures (*UNIT 6.2*).

   The use of nitrocellulose membranes is not recommended.

4. After transfer, fix the blot by immersing in 25 ml fix solution and incubate with gentle agitation (e.g., on an orbital shaker at 50 rpm) for 45 min at room temperature.

5. Wash the blot by incubating in 25 ml wash solution with gentle agitation for 10 min, room temperature. Repeat this wash step one additional time.

6. Oxidize the blot in 25 ml periodic acid solution with gentle agitation for 30 min.

7. Wash the blot in 25 ml wash solution with gentle agitation for 5 to 10 min. Repeat this washing step two additional times.

Visualize glycoproteins

8. Prepare fresh Pro-Q Emerald 300 staining solution by diluting the 50× Pro-Q Emerald 300 concentrate reagent 50-fold into Pro-Q Emerald 300 dilution buffer.

   For example, dilute 500 µl of 50× Pro-Q Emerald 300 concentrate reagent into 25 ml of dilution buffer to make enough staining solution for one 8 × 10–cm gel.

9. Incubate the blot in the dark in 25 ml Pro-Q Emerald 300 staining solution (step 8) while gently agitating for 90 to 120 min, room temperature.

   The signal can be seen after ~20 min and maximum sensitivity is reached at ~120 min. Staining overnight is not recommended.

10. Wash the blot with 25 ml wash solution for 15 min at room temperature. Repeat this wash one additional time. Do not leave the blot in wash solution for >2 hr, as the staining signal will start to decrease.

11. Allow the membrane to air dry.

12. Visualize the stain using a standard 300-nm UV epi-illuminator.

    The Pro-Q Emerald 300 stain has an excitation maximum at ~280 nm and an emission maximum near 530 nm.

    A UV transilluminator may also be used to visualize the glycoproteins, but this results in poorer detection sensitivity.

13. Document results before proceeding to the next step using a photographic camera or CCD camera with the appropriate filters to obtain the greatest sensitivity.

    Pro-Q Emerald dye signal will fade after SYPRO Ruby dye staining.

Visualize total protein

14. In order to counter-stain non-glycosylated proteins in the sample, pour the SYPRO Ruby protein blot stain solution into a small, clean plastic dish.

    For one or two standard-size mini-blots, use ~50 ml to 100 ml of staining solution; for larger blots, use 500 to 750 ml.

15. Place the air-dried blot face down onto the surface of the staining solution and gently agitate (e.g., on an orbital shaker at 50 rpm) for 15 min at room temperature.

16. After staining, rinse the blot in four changes of water for 1 min each to decrease background fluorescence.

17. Allow blots to air dry and visualize the stain using an appropriate method.

    The stained blot is best viewed on a standard 300-nm UV epi-illuminator, though stain will be visible using a 254-nm UV-C or 365-nm UV-A epi-illuminator. Blots may also be visualized using various laser scanners: 473-nm (SHG) laser, 488-nm argon-ion laser, or 532-nm (YAG) laser. Alternatively, use a xenon arc lamp, blue fluorescent light, or blue light–emitting diode (LED) source.

    Blots may be photographed by Polaroid or CCD camera. Use Polaroid 667 black-and-white print film and the SYPRO protein gel stain photographic filter (Molecular Probes). Exposure times vary with the intensity of the illumination source; for an f-stop of 4.5, ~1 to 3 sec should be required.

FLUORESCENT DETECTION OF GLYCOPROTEINS CONTAINING TERMINAL α-MANNOPYRANOSYL AND α-GLUCOPYRANOSYL RESIDUES ON ELECTROBLOT MEMBRANES

BASIC PROTOCOL 2

Lectins are sugar-binding proteins of nonimmune origin capable of agglutinating cells or precipitating glycoconjugates (Beeley, 1985). The specific interactions between labeled lectins and oligosaccharides form the basis of glycoprotein detection after separation by gel electrophoresis and transfer to membranes by electroblotting. Concanavalin A is a tetrameric protein, with each subunit containing a carbohydrate-binding site, a calcium ion–binding site, and a manganese–ion binding site. Concanavalin A binds specifically

to α-D-mannopyranosyl and α-D-glucopyranosyl residues, with substitutions or modifications at the C-3, C-4, or C-6 positions of the ring structure leading to greatly diminished binding (Beeley, 1985).

The Pro-Q Glycoprotein Blot Stain Kit with concanavalin A utilizes alkaline phosphatase-conjugated concanavalin A along with the fluorogenic substrate DDAO phosphate [9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate] to detect glycoproteins on nitrocellulose and poly(vinylidene difluoride) (PVDF) membranes. The detection procedure is similar to that of standard western (immuno)blotting. DDAO phosphate is rapidly converted to the long wavelength, red-fluorescent product, DDAO. DDAO absorbs maximally at either 275 nm or 646 nm and emits maximally at 659 nm. Consequently, the blots may be imaged using standard UV epi-illumination or with a laser-based gel scanner equipped with appropriate excitation source. The enzymatic amplification step greatly enhances the signal, allowing low nanogram detection of glycoproteins, a sensitivity on par with chemiluminescence detection methods. Pro-Q Glycoprotein Blot Stain kits with wheat germ agglutinin or with *Griffonia simpliifolia* lectin II (GS-II) allow detection of *N*-acetylglucosamine and sialic acid residues or terminal *N*-acetylglucosamine residues, respectively. The detection procedures for these lectins are quite similar to the concanavalin A method, and the same fluorogenic substrate, DDAO-phosphate is used in the kits.

Materials

Protein samples of interest
PVDF membrane
50% methanol
Wash solution II (see recipe)
Blocking solution (see recipe)
Pro-Q Glycoprotein Blot Stain Kit with Concanavalin A (Molecular Probes) containing:
  Concanavalin A, alkaline phosphatase conjugate (Con A-AP) stock solution (see recipe)
  DDAO phosphate stock solution (see recipe)
  Dimethylformamide (DMF)
  CandyCane glycoprotein molecular weight standards (see recipe), sufficient volume for ~20 gel lanes
Incubation buffer (see recipe)
10 mM Tris/1 mM $MgCl_2$, pH 9.5

Polystyrene staining dishes (e.g., weigh boat for minigel or larger container for larger gels)
Plastic wrap
UV epi-illumination and a digital or film camera, or a laser equipped with a 633-nm helium-neon laser or 635-nm diode laser source

Additional reagents and equipment for SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*), electroblotting procedures (*UNIT 6.2*), and SYPRO Ruby protein blot staining (see Alternate Protocol)

Run gel

1. Prepare the protein samples of interest (e.g., crude protein isolates, cell lysates, serum, partially purified plasma membranes) for SDS-polyacrylamide gel electrophoresis (*UNIT 6.1*).

   Typically, the protein sample is diluted to ~10 to 100 μg/ml with 2× sample buffer, heated for 4 to 5 min at 95°C, and 5 to 10 μl of diluted sample is then applied per gel lane for 8× 10–cm gels. Larger gels require proportionally more material.

For convenience, CandyCane glycoprotein molecular weight standards may also be applied to a lane or two. Typically, 2 µl of this standard is diluted in 6 µl of sample buffer and heated in the same manner as the samples to be characterized. These standards contain a mixture of glycosylated and non-glycosylated proteins ranging from 14 to 180 kDa in molecular weight. The standards serve as molecular weight markers and as alternating bands of positive and negative controls for glycoprotein and total protein detection. Each protein is present at 0.5 mg/ml.

2. Separate proteins by SDS-polyacrylamide gel electrophoresis using standard methods (*UNIT 6.1*).

   The procedure is optimized for gels that are 0.5- to 1-mm thick.

Blot proteins

3. After electrophoresis, transfer the proteins to PVDF membrane using standard electroblotting procedures (*UNIT 6.2*).

   The use of nitrocellulose membranes is not recommended.

4. *Optional*: Stain blots with SYPRO Ruby protein blot stain at this point to visualize the total protein pattern and to verify that the blotting procedure was successful. Follow the staining procedure described in Alternate Protocol, steps 14 to 17.

   Total protein staining must be performed prior to lectin blotting as the blocking mixture will produce very high background on the blot. Since SYPRO Ruby protein blot stain is washed off during the subsequent lectin blotting process, it is important to document staining results before continuing with the procedure.

5. If the PVDF blot is dry, briefly hydrate in 50% methanol and incubate in wash solution II for 10 min at room temperature. Repeat the wash step for a total of three washes.

Visualize glycoproteins

6. Incubate the blot in blocking solution for 1 to 2 hr at room temperature.
7. Briefly pellet any potential protein aggregates in the Con A-AP stock solution by microcentrifugation. Using the supernatant only, dilute the Con A-AP stock solution 2000-fold by adding 5 µl to 10 ml of incubation buffer for a final concentration of 1 µg/ml. Remove the blocking solution that the blot is immersed in and incubate the blot with Con A-AP solution for 1 hr at room temperature.
8. Remove the diluted Con A-AP solution and wash the blot in blocking solution four times for 10 min each at room temperature.
9. Perform two final washes in wash solution II for 5 min each at room temperature.
10. Dilute the DDAO phosphate stock solution 1000-fold into 10 mM Tris/1 mM $MgCl_2$, pH 9.5, for a final concentration of 1.25 µM.

    Approximately 1 ml of the DDAO phosphate staining solution will be needed for an 8× 10–cm blot. Note that DDAO phosphate is unstable when stored at room temperature as an aqueous solution. Always make up the DDAO phosphate staining solution just prior to use.

11. Incubate the blot in freshly prepared DDAO phosphate staining solution.

    The staining step may be performed either face up or face down, depending on the configuration of the imaging instrumentation being used. If using UV epi-illumination or a laser scanner with a light source that illuminates from above the imaging bed, stain the blot face up. For laser scanners with light sources that illuminate the blot from below the imaging bed, stain the blot face down.

12. Using powder-free gloves, cut a piece of plastic wrap to the size of the blot. For face-up staining, place the blot on the plastic wrap and pipet 1 ml of DDAO phosphate staining solution onto the blot. For face-down staining, pipet 1 ml of the DDAO

phosphate staining solution onto the plastic wrap and lay the blot face down onto the solution, being careful not to trap air bubbles.

The time required for optimal staining must be determined empirically because the substrate turnover rate depends on the amount of glycoprotein on the blot. Generally, a 5- to 20-min incubation is sufficient, but overnight incubation is permissible. Do not wash the blot after staining as this will cause extensive loss of signal. The blot may be air-dried, however.

13. Visualize the fluorescent DDAO product using either UV epi-illumination and a digital or film camera, or using a laser equipped with a 633-nm helium-neon laser or 635-nm diode laser source. For UV epi-illumination, place the blot, signal side up, on a flat surface. For highest sensitivity and lowest background, use a UV-blocking filter, such as the SYPRO gel stain photographic filter. Long-pass filters with a cutoff at ~630 nm are ideal for CCD-cameras. For laser scanners, place the blot, signal side down, on the scanner bed. For highest sensitivity, match the light sources and filters of the instrument as closely as possible to the absorbance maximum (646 nm) and emission maximum (659 nm) of DDAO.

14. If desired, the Con A-AP complex can be stripped off of the blot and the blot reprobed with another lectin-AP complex or an antibody-AP complex. To strip, incubate the blot in stripping buffer for 40 min at 50°C with gentle agitation. Then, wash the blot in wash buffer two times for 5 min each at room temperature.

REAGENTS AND SOLUTIONS

Use deionized or distilled water in all recipes and protocol steps. For common stock solutions see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Blocking solution

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2A*)
150 mM NaCl
0.2% (v/v) Tween 20
0.25% (w/v) Mowiol 4-88 (Calbiochem or VWR)
Store up to 6 months at room temperature

The use of Mowiol 4-88 in the Blocking buffer is not essential, but does decrease background staining and improves detection sensitivity. As an alternative to Mowiol 4-88, 0.5% (w/v) bovine serum albumin or 4% (w/v) gelatin (high purity, e.g., TopBlock from Juro Supply) may be used.

CandyCane molecular weight standards

For a standard lane on an 8 × 10–cm gel, dilute 0.5 µl of the standards with 7.5 µl of 2× sample buffer (see recipe) and vortex. This will result in ~250 ng of each protein per lane, a sufficient amount for detection of the glycoproteins by the Pro-Q Emerald 300 stain. For large 16 × 18–cm gels, double the amount of standard and buffer used. Store up to 6 months at room temperature.

Con A-AP stock solution

Prepare a 2 mg/ml stock solution of Con A-AP by dissolving the vial contents in 250 µl deionized water and add 2 mM sodium azide. The stock solution is stable for at least 6 months when stored undiluted at 4°C. Do not freeze.

DDAO phosphate stock solution

Add 200 µl DMF to the vial containing 250 µg DDAO phosphate {[9H-(1,3-dichloro-9, 9-dimethylacridin-2-one-7-yl] phosphate, diammonium salt} to make 1.25 mg/ml stock solution. Store the stock solution at –20°C, desiccated and protected from light. When properly stored, the stock solution should be stable for at least 6 months. When the solution turns a blue color, the substrate has broken down and is no longer usable. Prepare the working solution fresh.

Fix solution

Prepare a solution of 50% methanol and 50% deionized water. Store up to 6 months at room temperature.

One 8 × 10–cm gel will require ~100 ml of fix solution.

Incubation buffer

Prepare blocking solution (see recipe) with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Store up to 6 months at room temperature.

Periodic acid solution

Add 250 ml of 3% (v/v) acetic acid to the bottle containing the periodic acid (oxidizing solution) and mix until completely dissolved. Store up to 6 months at room temperature.

Sample buffer, 2×

100 mM Tris·Cl, pH 6.8 (*APPENDIX 2A*)
20% (v/v) glycerol
4% (w/v) sodium dodecyl sulfate
0.1% (w/v) bromophenol blue
Store up to 6 months at room temperature

Wash solution

Prepare a solution of 3% (v/v) glacial acetic acid in water. Store up to 6 months at room temperature.

One 8 × 10–cm gel will require ~400 ml of wash solution.

Wash solution II

Prepare a solution of 50 mM Tris·Cl, pH 7.5/150 mM NaCl. Store up to 6 months at room temperature.

COMMENTARY

Background Information

The analysis of protein glycosylation is readily accomplished by polyacrylamide gel electrophoresis (Koch and Smith, 1990; Packer et al., 1997; Taverna et al., 1998; Koketsu and Linhardt, 2000). However, relatively few high-sensitivity methods have been developed to reliably detect oligosaccharide residues covalently attached to proteins for visualization in polyacrylamide gels or on electroblot membranes (Packer et al., 1997; Packer et al., 1999; Koketsu and Lindhardt, 2000). Perhaps the most common procedure to visualize glycoproteins reported in the literature entails detection by periodic acid/Schiff (PAS) staining using the colorimetric acid fuchsin dye. A major limitation of this method is that detection sensitivity is poorer than Coomassie Blue staining, rendering the technique obsolete for modern high-sensitivity proteomics investigations. Other methods in use include PAS-labeling with digoxigenin hydrazide followed by immunodetection with anti-digoxigenin antibody conjugated to alkaline phosphatase, or PAS-labeling with biotin hydrazide, followed by detection with horseradish peroxidase or alkaline phosphatase conjugated to streptavidin (Packer et al., 1995). Lectins are commonly employed to detect certain structural subclasses of glycoproteins by methods similar to those employed in immunoblotting (Koketsu and Lindhardt, 2000). All of these methods require that pro-

teins be electroblotted to membranes first and many glycoproteins transfer relatively poorly. In addition, detection of glycoproteins after electroblotting is very time consuming compared with direct detection in gels. A recently developed approach to the detection of glycoproteins relies upon the utilization of a new fluorescent hydrazide, Pro-Q Emerald 300 dye, that is affixed to glycoproteins using a standard PAS conjugation mechanism (Steinberg et al., 2001). The glycols present in glycoproteins are initially oxidized to aldehydes using periodic acid. The dye then reacts with the aldehydes on the glycoproteins to generate the fluorescent conjugate. A reduction step with sodium metabisulfite or sodium borohydride is not required to stabilize the resulting conjugate.

Critical Parameters

All stock solutions should be prepared using deionized water (dH_2O) having a resistance of at least 18 MΩ. All stock solutions may be stored for up to 6 months at room temperature, except when specifically indicated. Dilution of the DDAO phosphate stock solution or the Pro-Q Emerald 300 dye solution should be performed immediately prior to their use in the staining protocols. Both reagents are unstable when stored at room temperature as aqueous solutions. The staining methods outlined in this unit are highly sensitive and it is critical that all glassware and staining dishes be scrupulously clean. Gels and blots should never be touched or otherwise manipulated using bare hands. Always wear powder-free latex gloves when handling gels and blots during all staining procedures for the fluorescence detection of glycoproteins.

Troubleshooting

Should the detection sensitivity obtained using the cited fluorescence methods be suboptimal, there are two potential sources for the problem, either instrumental or chemical. With respect to the imaging instrument, it is important to clean the surface of the transilluminator after each use with deionized water and a soft cloth (e.g., cheesecloth). Otherwise, fluorescent dyes can accumulate on the glass surface and cause a high background fluorescence. The polyester backing on some pre-cast gels is highly fluorescent. For maximum sensitivity using a UV transilluminator, the gel should be placed polyacrylamide side down and an emission filter used to screen out the blue fluorescence of the plastic. For UV detection of fluorophores, a 300-nm UV-B transilluminator with six 15-watt bulbs is recommended. Excitation with different UV light sources, such as a simple hand held UV lamp will not provide the same level of detection sensitivity as a full-fledged transilluminator. For all three procedures described in this unit, using a Polaroid camera and Polaroid 667 black-and-white print film, the highest sensitivity is achieved with a 490-nm long-pass filter, such as the SYPRO protein gel stain photographic filter (S-6656; Molecular Probes). Gels are typically photographed using an f-stop of 4.5 for 2 to 4 sec, using multiple 1-sec exposures. Using a CCD camera, images are best obtained by digitizing at ~1024 × 1024–pixels resolution with 12-, 14-, or 16-bit gray-scale levels per pixel. A 520-nm long-pass filter is suitable for visualizing Pro-Q Emerald dye, while a 580-nm long-pass filter is appropriate for detection of DDAO. A CCD camera-based image analysis system can gather quantitative information that will allow comparison of fluorescence intensities between different bands or spots. Using such a system, the Pro-Q Emerald dye and the DDAO dye have a linear dynamic range of three orders of magnitude.

A potential problem associated with the Pro-Q Emerald 300 glycoprotein gel stain is nonspecific labeling of non-glycosylated proteins. The most common source of this problem is the presence of residual SDS in the polyacrylamide gel. Adding an extra fixation step to the procedure should prevent its occurrence. The author finds that an overnight fixation step for two-dimensional gels is advisable. When detecting glycans using Pro-Q Emerald 300 glycoprotein gel stain, it is prudent to run control gels in which the periodate oxidation step has been omitted. Similar precautions are advisable when evaluating results using other glycoprotein detection methods. This avoids erroneous interpretation of results arising from low levels of noncovalent binding of dye molecules or confusion arising from the inherent fluorescence of certain high-abundance proteins in the gel profile.

The most common problem encountered using the Pro-Q glycoprotein detection kit with concanavalin A is poor signal intensity. This is usually due to decomposition of the DDAO phosphate stock solution. When the stock solution appears by eye to be a blue color, the substrate has broken down and is no longer usable.

Table 6.8.1 Comparison of Commonly Used Glycoprotein Detection Methods for Polyacrylamide Gels and Electroblot Membranes

Detection method	Time required (hr)	Number of steps	Detection sensitivity (1) α1-acid glycoprotein (40% CHO)[a] (2) glucose oxidase (12% CHO)[a] (3) avidin (7% CHO)[a]		Assets (+) or liabilities (–)
			Gels	*Blots*	
Acid fuchsin sulfite (pararosaniline)	5-6	7	(1) 75 ng (2) 150 ng (3) 150 ng	20 ng 75 ng 75 ng	(+) short procedure (+) can use either on blots or in gels (–) poor sensitivity
Biotin hydrazide/ streptavidin-HRP Luminol detection reagents	6	11	(1) na (2) na (3) na	18-37 ng 37 ng 150 ng	(–) signal fades over time, optimal image, 20-30 min (–) cannot save and reimage blots
Biotin hydrazide/ streptavidin-alkaline phosphatase NBT/BCIP solution	5-6	11	(1) na (2) na (3) na	2 ng 5-9 ng 18-37 ng	(+) good sensitivity (+) can save and reimage blots (–) long procedure (–) cross reaction with carbonic anhydrase
Pro-Q Glycoprotein Detection Kit with Con-A alkaline phosphatase (see Basic Protocol 2)	4	5	(1) na (2) na (3) na	not detected <15.6 ng 15.6 ng	(+) can save and re-image blots (+) can strip and reprobe (+) can post-stain with total protein stains (+/–) stains specific subsets of glycoproteins (–) long procedure
Dansyl hydrazine	4	9	(1) 1.25-2.5 ng (2) 1.25-2.5 ng (3) 16-19 ng	not tested	(+) inexpensive (–) requires longer exposure for competitive brightness (–) low-level non-specific detection of unglycosylated proteins (–) requires hot, acidified DMSO
Digoxigenin-3-*O*-succinyl-ε-aminocaproic acid hydrazide/ Anti-digoxigenin-alkaline phosphatase. Stain with NBT/x-phosphate	5-6	11	(1) na (2) na (3) na	2 ng 5-9 ng 18-37 ng	(+) good sensitivity (+) can save and reimage blots (–) long procedure (–) cross reaction with carbonic anhydrase
Pro-Q Emerald 300 Dye (see Basic Protocol 1 and Alternate Protocol)	2 (blots) 4 (gels)	7	(1) 300 pg (2) 300 pg (3) 1-2 ng	2 ng 18 ng 9 ng	(+) can use either on blots or in gels (+) great sensitivity (+) can save and re-image blots (+) short procedure (+) can counterstain unglycosylated proteins with SYPRO Ruby dye

[a]Abbreviations: CHO, carbohydrate; na, not applicable.

Anticipated Results

The performance characteristics of the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, Pro-Q Emerald 300 Glycoprotein Blot Stain Kit, and Pro-Q Glycoprotein Detection Kit with Concanavalin A are summarized in Table 6.8.1 and contrasted with alternative glycoprotein detection technologies. The fluorescence-based methods permit detection of low-nanogram amounts of glycoprotein with a dynamic range of quantitation that encompasses three orders of magnitude of glycoprotein abundance. Pro-Q Emerald 300 dye may be used to detect a variety of glycoconjugates in addition to glycoproteins, such as bacterial lipopolysaccharides (LPS) and glycogen. Detection sensitivity for chondroitin 4-sulfate, however, is ~3000-fold poorer than glycogen or LPS, with limits of detection in the vicinity of 16 μg of applied material. This is not unexpected as glycosaminoglycans such as chondroitin sulfate, hyaluronic acid, and keratan sulfate are known to stain poorly by conventional PAS procedures. Concanavalin A specifically binds to nonsubstituted and 2-*O*-substituted α-mannosyl residues and thus detects fewer glycoproteins than the Pro-Q Emerald 300 dye. For example, α1-acid glycoprotein is not detected by concanavalin A. Similarly, glycoproteins such as ovomucoid (28 kDa) and ovotransferrin (76 kDa) are not effectively detected by concanavalin A. The differences in staining specificity between the Pro-Q glycoprotein detection kit with concanavalin A, the Pro-Q glycoprotein detection kit with wheat germ agglutinin and the Pro-Q Emerald 300 glycoprotein stain kits can be exploited in defining structural features of glycans on glycoproteins.

Time Considerations

The time considerations and number of steps required to detect glycoproteins using the Pro-Q Emerald 300 Glycoprotein Gel Stain Kit, Pro-Q Emerald 300 Glycoprotein Blot Stain Kit, and Pro-Q Glycoprotein Detection Kit with Concanavalin A are summarized in Table 6.8.1 and contrasted with alternative glycoprotein detection technologies. The methods can be completed in ~2 to 4 hr and require 5 to 7 steps to complete. This compares favorably with other methods that may require as much as 6 hr and 11 steps to complete.

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Describes optimized methods for protein identification by matrix-assisted laser desorption time-of-flight mass spectrometry after staining gels with SYPRO Ruby dye.

Internet Resources

http://www.cbs.dtu.dk/databases/OGLYCBASE/

O-GLYCBASE; a database of 198 glycoprotein entries with experimentally verified O-glycosylation site information.

http://www.glycosuite.com

GlycoSuite; a relational database that curates information from the scientific literature on glycoprotein derived glycan structures, their biological sources, the references in which the glycan was described, and the methods used to determine the glycan structure.

http://www.expasy.ch/tools/glycomod/

GlycoMod; a software tool designed to find all possible compositions of a glycan structure from its experimentally determined mass.

http://www.probes.com

Molecular Probes commercial Web site containing information about fluorescence detection technologies, including glycoprotein, total protein, lipopolysaccharides, and nucleic acids.

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Digital Electrophoresis Analysis

Gel electrophoresis has become a ubiquitous method in molecular biology for separating biomolecules. This prominence is the result of several factors, including the robustness, speed, and potentially high throughput of the technique. The results of this method are traditionally documented using silver halide–based photography followed by manual interpretation. While this remains an excellent method for qualitative documentation of single-gel results, digital capture offers a number of significant advantages when documentation requires quantitation and sophisticated analysis. Digital images of gel electropherograms can be obtained rapidly using an image-capture device, and the images can be easily manipulated using image analysis software.

REASONS FOR DIGITAL DOCUMENTATION AND ANALYSIS

There are several reasons to consider digital documentation and analysis of electrophoresis results. These justifications can usually be categorized into issues of ease of handling, accuracy, reproducibility, and cost.

Ease of Handling

A major advantage of the digital revolution has been in storage and retrieval of information. Storage in notebooks and filing cabinets previously meant that searching for specific data or experiments was a tedious manual process. With digital information, modern search engines can quickly find specific information in a fraction of the time usually required for a manual search. Making backup copies of nondigital data can be difficult, expensive, and time-consuming since it requires copying, retyping, or photographic reproduction. Copies of digital data can be generated more easily and at reduced costs.

Manipulation of information is also easier when it is in a digital format. While the cut-and-paste analogy comes from physical documentation, it takes on a new perspective when applied digitally. Electrophoresis images can be resized, cropped, and inserted into reports. Data can be passed to spreadsheets and statistical packages for analysis and later insertion into notebooks and reports. These reports can be passed out via the Internet to colleagues throughout the world. A single individual can do all this in a few hours.

Digital analysis also provides an easier method for handling the data when comparing large numbers of results or large numbers of separate experiments. Research that requires comparing the banding patterns on 1000 gels containing 50 lanes each can be an undertaking of heroic proportions if the analysis is performed manually. Database software can dramatically speed the analysis and handle the more mundane tasks, leaving the researcher free to interpret the data.

Accuracy

The human eye is an extremely versatile measuring instrument. It can handle light intensities covering a range of nearly nine orders of magnitude and is sensitive to a fairly wide spectrum of light (Russ, 1995). Yet the eye cannot accurately and reproducibly quantitate density and patterns, nor can it deal with large numbers of bands or spots. Accuracy of measurement is a primary reason for using digital analysis on electrophoretically separated proteins and nucleic acids. Two categories of accuracy are key to digital analysis: positional accuracy, which is important for mobility determinations such as molecular weight, and quantitative accuracy.

Positional accuracy is based on both resolution of the recording medium and measuring accuracy. Silver halide–based recording has a theoretical resolution based on ~2000 imaging elements (silver grains) per inch. Measurement traditionally occurs using a ruler, with an accuracy of ~20 to 40 elements (50 to 100 elements per inch). In comparison, typical digital systems have 200 to 600 picture elements (pixels) per inch. The advantage that digital systems have is in measuring accuracy, which can occur at the level of a single imaging element.

Quantitative accuracy is also an issue. The amount of material represented by a band or spot is difficult to determine accurately from an image of a gel unless it is a digital image. On a digital image, the amount present is directly correlated with the derived volume of the band or spot—the volume is calculated using the intensity values of the pixels within the object.

Reproducibility

Any technique or measurement is only as good as its ability to be faithfully replicated. With software-defined routines, measurements

Contributed by Scott Medberry and Sean Gallagher
Current Protocols in Cell Biology (2002) 6.19.1-6.19.14
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are performed in the same manner every time. Allowing the computer to do repetitive tasks and complicated calculations minimizes the chance for individual errors. This does not imply that such measurements are correct, just that they are reproducible. An incorrect routine or algorithm can also invalidate data.

Cost

A consideration when evaluating any laboratory method is cost. Digital electrophoresis analysis equipment can be expensive. In many cases, however, it offers the only method for achieving acceptable analysis performance. In other cases, equal performance can be achieved using silver halide technology. However, traditional photography can also be expensive when the costs of consumable supplies such as film and developers, as well as other expensive requirements such as developing tanks and dark rooms, are included. Often, digital methods can be a good choice when all costs are considered.

KEY TERMS FOR IMAGING

There are several specialized terms encountered during digital image analysis. The most commonly encountered are contrast, brightness, gamma, saturation, resolution, and dynamic range. They describe controls on how the light detectors report a range of light intensities. Below is a brief description of each.

Contrast

Contrast describes the slope of the light intensity response curve. An increase in the contrast increases the slope of the curve. The result is a more detailed display over a narrowed range of intensities with less detail in the remaining portions of the intensity range. This is depicted in Figure 6.9.1A and 6.9.1B, where a normal, unadjusted image and a contrast-adjusted image are displayed, respectively. The contrast was increased on midrange intensity values in Figure 6.9.1B to highlight band intensity differences at the expense of background information. Images with a narrow range of informative intensities can benefit from increasing the contrast, since that effectively increases the scale and improves detection of minor differences in intensity. Contrast settings should be lowered if information is being lost outside of the contrast range. For example, in Figure 6.9.1B, loss of background information between peaks indicates that this image should not be used for quantitation.

Brightness

While brightness can have many different definitions, only one will be considered here. Brightness shifts the light intensity response curve without changing its slope as is shown in Figure 6.9.1C. Another name for brightness is black level, since it is commonly used to control the number of black picture elements (pixels) in an image. Incorrect brightness levels can lead either to high background and potential image saturation or, as is illustrated in Figure 6.9.1C, to a total loss of background information and partial loss of band information.

Gamma

Nonlinear corrections are often applied to images to compensate for how the eye perceives changes in intensity, how display devices reproduce images, or both. The most common correction is an exponential one, with the exponent in the equation termed the gamma. A typical gamma value for camera-based systems is 0.45 to 0.50, and is illustrated in Figure 6.9.1D. This is a compromise value that compensates for the 2.2 to 2.5 gamma present in most video monitors and the print dynamics of most printers. Since it is a nonlinear correction, special care must be taken if quantitation is desired. Unless directed to by the manufacturer, gamma values other than 1.0 should be avoided when quantitating. More information on gamma correction can be found on Poynton's Gamma FAQ (*www.inforamp.net/~poynton/Poynton-color.html*).

Dynamic Range

Dynamic range describes the breadth of intensity values detectable by a system and is usually expressed in logarithmic terms such as orders of magnitude, decades, or optical density (OD) units. A large dynamic range is important when trying to quantitate over a wide range of concentrations. The most accurate quantitation occurs in the linear part of the dynamic range, which is usually not the complete dynamic range of the system. An additional consideration is the dynamic range of the visualization method. Many popular visualization methods have linear dynamic ranges of 1 to 2.5 orders of magnitude. An imaging system with greater dynamic range analyzing the results of such a visualization method will not improve the dynamic range.

Saturation

Saturation occurs when a detector or visualization method receives input levels beyond

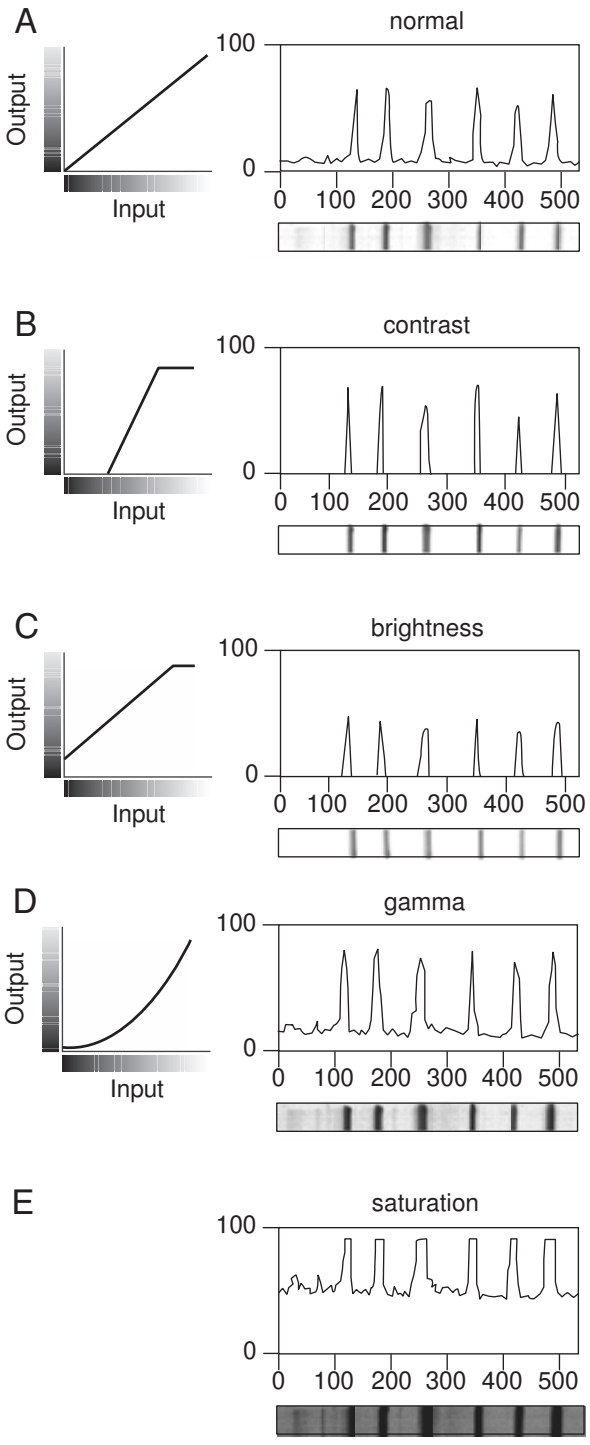

Figure 6.9.1 Examples of how altering image capture settings affects the image and the analysis. The graph on the left displays the light intensity response curve used for image capture while the image and resulting lane profile on the right display how the setting affects the image. The lane profile displays pixel position versus normalized pixel intensity (**A**). In this case, the output has not been altered, giving a straight line with a slope of 1 on the response curve. (**B**) The image acquisition was adjusted to increase the contrast of the displayed image. Although useful for images with a narrow range of informative intensity values, increasing the contrast can lead to a loss of low and high values. (**C**) Decreasing the brightness reduces peak values but also leads to a loss of the weak bands and original background. (**D**) Gamma adjusts raw data to appear more visually accurate. Note that this leads to a loss of fidelity between the adjusted image and the original. (**E**) Saturation indicates that the detector is reporting its maximum value or that the dynamic range for the visualization method has been exceeded.

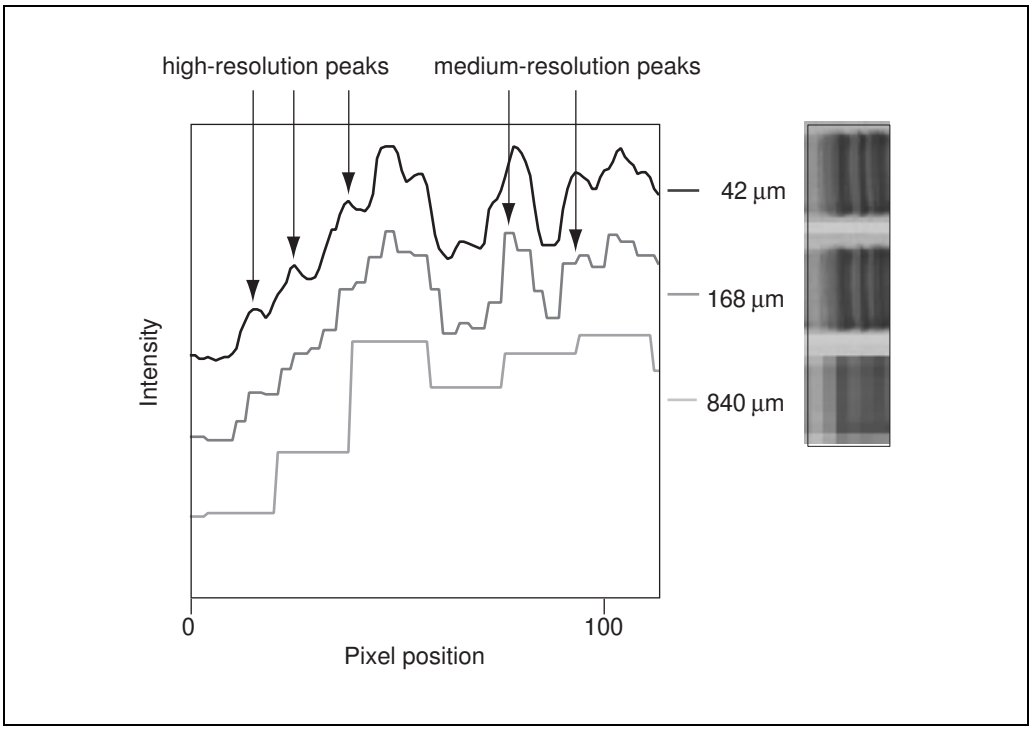

Figure 6.9.2 The effect of spatial resolution on the ability to detect closely spaced objects. Whole-cell protein lysates from *E. coli* were separated using SDS-PAGE and visualized with Coomassie blue staining. An image was captured at 42 μm (600 dpi), 168 μm (150 dpi), and 840 μm (30 dpi) from a segment of the lane, and a lane profile was generated for each image. The lane profiles have offset intensities to allow for comparison. Only major bands can be detected with the low-resolution image (840 μm); at higher resolutions more bands are detectable.

the maximum end of the dynamic range. This results in a loss of detail and quantitative information from those data points that are saturated. For fluorescent and luminescent samples, reduction in the sampling time can sometimes correct saturation problems. Optical density-based visualization techniques can also generate saturated images, as is illustrated in Figure 6.9.1E; this can sometimes be avoided with longer sampling times or increased detection source intensities. More often, it will be necessary to perform another electrophoresis with more dilute samples or to alter the visualization process to generate a less optically dense material.

Resolution

Resolution is the ability of a system to distinguish between two closely placed or similar objects. Three types of resolution are important for analysis—spatial resolution, intensity resolution, and technique-dependent resolution.

Spatial resolution is the ability to detect two closely placed objects in one-, two-, or three-dimensional space. It is most accurately described as the closest distance two objects can be placed and still be detected as separate objects. In practice, it is often defined nominally in terms of the number of detectors per unit area such as dots per inch (dpi) or the number of detectors present in total or in each dimension such as 512 × 512 (262,144 total detectors). Actual resolution is less than half the nominal resolution due to the need for two detectors for every resolvable object (one for the object and one for the separation space) and the effects of optical resolution. Figure 6.9.2 demonstrates how spatial resolution can affect detection of objects. The 42-μm resolution image allows detection of closely spaced bands, the 168-μm resolution image detects fewer bands, and the 840-μm resolution image detects only major bands. For instruments with on-line detection systems, a pseudo spatial resolution is often reported in units of time from the start of the separation or the time interval between two objects crossing the detection path.

Intensity resolution is the ability to identify small changes in intensity. It is a function of both the dynamic range of a detector and the number of potential values that detector can report. Greater dynamic range decreases the intensity resolution of a given detector. The number of potential values a detector reports is

described by its bit depth. An 8-bit detector can report 256 (2^8) different possible values, while a 12-bit detector can report 4,096 (2^{12}) values, and a 16-bit detector can report 65,536 (2^{16}) values. The higher the bit depth, the greater the intensity resolution.

Technique-dependent resolution directly affects the spatial and intensity resolution. Electrophoretic separation techniques that generate overlapping objects or that have object separation distances shorter than the spatial resolution will fail to provide reliable data. Many factors, including the amount of sample loaded, gel pore size, buffer constituents, and electrophoresis field strengths, can dramatically affect separation and resolution of biomolecules. Likewise, detection methods that can only generate a small range of discrete intensity values will not benefit from systems with improved intensity resolution.

IMAGE CAPTURE

Devices

Capturing digital images involves a detection beam or source, a sensor for that beam or source, and some method of assembling a two-dimensional image from the data generated. Most systems use a light source for detection. The light wavelengths used range from ultraviolet (UV) to infrared (IR) and can be broad spectrum or narrow wavelength. Broad-spectrum detection is more versatile since it can often be used for more than one detection wavelength. However, when compared to narrow-wavelength sources such as lasers, broad-spectrum detection suffers from reduced sensitivity and reduced dynamic range. While many types of light sensors have been used, including charge-coupled devices (CCDs), charge-injection devices (CIDs), and photon multiplier tubes (PMTs), technology advances in CCDs have led to their dominance. CCDs are semiconductor imaging devices that convert photons into charge. This charge is then read and converted into a digital format via an analog-to-digital converter (ADC).

The method of image assembly depends on the light source and detector geometry. One method is to capture the image all at once using a two-dimensionally arrayed CCD detector similar to the detectors found in digital and video cameras. Typically a camera-type sensor is paired with a light source that evenly illuminates the sample. This same sensor is often used with fluorescent and chemiluminescent detection methods, as its ability to detect light continuously over the entire sample reduces image capture times. Another method of image assembly is to capture the image a line at a time. This typically involves a linearly arrayed CCD scanning slowly across the sample in conjunction with the detection beam of light. The data from each line is then compiled into a composite image. Spatial resolution in this method can be significantly better on large-format samples than the resolution of a camera-based system. This method is also advantageous when OD-based detection is used, since the more focused light beam is usually of higher intensity and can penetrate denser material. A third method of image assembly is to use a point light source and single-element detector on each point on a sample. The image is then compiled from each point sampled. This method is slower than the others but can offer extremely high resolution and sensitivity. A fourth commonly encountered method is that of generating a pseudoimage of electrophoresis results through the use of a finish line type of detection system. This is comprised of a light source positioned at the bottom of the gel (i.e., the end opposite of the site of sample loading) and light detectors positioned next to each lane to detect the transmitted light or emitted fluorescence. A lane trace is generated using time on the *x* axis and light intensity on the *y* axis. The pseudoimage is then generated from this data (Sutherland et al., 1987).

Capture Process

Prior to image capture, electrophoretic separation and any visualization steps are performed. To calibrate the separation process, standards are usually run at the edges of the gel and often at internal positions. If quantitation of specific proteins or nucleic acids is to be performed, a dilution series of standards with similar properties to the experimental samples should also be included. After separation, the protein or nucleic acid is visualized if necessary. Visualization can include binding of a fluorochrome or chromophore such as Coomassie blue, precipitation of metal ions such as copper, silver, or gold, enzymatic reactions, and exposure of film or phosphor screens to radiant sources. These methods can be grouped based on the type of detection into optical density, fluorescence, chemiluminescence, and radioactivity. The suitability of popular detection devices with these methods is described in Table 6.9.1. Once visualization has occurred, image capture consists of the following steps: previewing the image while

Table 6.9.1 Compatability of Popular Image-Capture Devices with Common Visualization Methods[a]

Visualization method	Image-capture device				
	Silver halide photography	CCD camera	Desktop scanner	Storage phosphor	Fluorescent scanner
Optical density[b]	+	+	++	−	−
Fluorescence	+	+	−	−	++
Chemiluminescence	+	++	−	±	−
Radioactivity	+	−	−	++	−

[a]The device with the highest sensitivity and greatest dynamic range for a visualization method is marked with a ++, other devices that can detect this visualization method are indicated with a +, and devices that are not suitable for a visualization method are indicated with a −. A ± indicates that only some devices of this type can be used with this visualization method.

[b]Optical density methods include Coomassie blue staining.

adjusting capture parameters, capturing the image, and saving the image for later analysis.

During the preview process, capture parameters are optimized for data content and for ease and rapidity of later processing steps. Typically, the first step is to place the sample so that when the image is captured, the rectangular edges of the gel are horizontal and vertical on the monitor and any lanes are either horizontal or vertical. Since band and spot detection will be much easier if the image is properly oriented, this eliminates the need to later rotate the image digitally. Image rotation is time-consuming and can result in spatial linearity errors (a change in the size and shape of objects in image) caused by rectangular image-capture device geometries. The next step for camera-based systems is to adjust magnification and to focus the sample image. For thicker samples, it might be necessary to reduce the aperture on camera-based systems to get a sufficient depth of field to focus the entire sample. Often at this point image imperfections—e.g., dust, liquid, or other foreign objects that will detract from later analysis—are detected, and they need to be removed. Next, image intensity is set. Within the area of interest on the image, band or spot peaks should have values less than the maximum saturated value, and the background should have nonzero values. This is usually accomplished through adjustment of the light-source intensity or the sensor signal integration. If the device allows precapture optimization of other parameters such as spatial resolution, contrast, brightness, gain, or gamma, they are adjusted next. Note that this only applies to controls that affect the response of the sensor or processing of the image prior to a data reduction step and not to controls that affect the image at later stages. The latter process can enhance visualization of specific features but is best left to adjustments in look-up tables (LUTs) in later analysis steps rather than during image capture, since there is a risk of data loss during postacquisition image processing. LUTs are indexed palettes or tables where each index value corresponds to color or gray-scale intensity values present in an image. Many image analysis programs alter LUTs instead of image values directly, since it both is faster and does not change the original image data.

Once all the capture parameters are optimized, the image capture process is initiated. This might take less than a second for images captured with camera-based detectors and up to hours for scanning single-point detectors. When the image has been captured, it should be carefully examined for content. It should fully capture the area of interest and the parameters should have been set so that all necessary information is detectable. Furthermore, it should be in a form that will allow for easy analysis. Extra time spent optimizing the capture parameters will often result in a reduction in total image analysis time and in an increase in data quality. When the best possible image has been captured, it often contains information outside the area of interest. While this is unlikely to cause problems with later analysis, it is often advantageous to crop the image so that the only portion that is saved contains the area of interest. This reduces the amount of disk space necessary to store the image, and the image usually will load and analyze faster with the analysis software.

The last step in image capture is to save the image. Several options are available at this point, including choosing which location to

save the image at, what file type or format to use, and whether to use some form of compression.

The location where the image is saved is not as trivial a question as it might seem if the image will need to be transferred to another computer at some point. File sizes can easily exceed 15 megabytes on high-resolution images. This is a manageable size for hard drives but exceeds current floppy drive sizes by an order of magnitude. There are software utilities available that will subdivide files into disk-size chunks and then reassemble them at the next computer, but this is an inconvenient and slow method. If the computer used to help capture the image is connected to a network, the image files can easily be transferred this way or potentially saved on a central server. Alternatively, several types of high-capacity removable media are available (e.g., Zip or Jazz). This usually requires the installation of additional hardware onto two or more computers but does make backing up data easier.

Since image files can be very large, compression techniques are sometimes used to reduce disk space requirements. Compression algorithms use several methods, typically by replacing frequent or repetitive values or patterns with smaller reference values and by replacing pixel values with the smaller difference values describing the change in adjacent pixels. When the file is later decompressed, the compressed values are then replaced with the original information. Not all images compress equally, with simple images containing mostly repetitive motifs compressible by ≥90%, while complex images will benefit much less from compression. Because compression is a much slower method of saving files and will not benefit every file, compression is not used to save all files. Several different forms of compression are available but are separable into two main classes, lossless and lossy. Lossless methods faithfully and completely restore the image when it is decompressed (no loss of data) but offer only moderate file compression; compression values range from ~10% to 90%, depending on the image. Examples of lossless compression include Huffman coding (Huffman, 1952), RLE (Run Length Encoding), and LZW (Lempel, Ziv, and Welch; Welch, 1984). In comparison, lossy methods such as JPEG (Joint Photographic Experts Group), MPEG (Moving Picture Experts Group), or fractal compression schemes can reach compression values of ≥98% (Russ, 1995). The trade-off is that not all information from the original file is recovered during decompression. Lossy compression is sometimes necessary for applications with extremely large image files such as real time video capture, but it usually represents an unacceptable loss of data if used with electrophoresis image capture.

Many different file types have been developed to store digital images. Some of these file types are proprietary or hardware specific. For example, PICT is a Macintosh format and BMP is a PC-compatible format. Each file type has its own structure. Some types do not allow compression, for others it is optional, and for some it is mandatory. File types vary in the types of images they support, particularly in the number of colors or gray levels. Below is a brief description of a few of the more prevalent file types.

TIFF (Tagged-Image File Format) is one of the most commonly used formats. It is particularly versatile since it is an open format that can be modified for specific applications. One reason for its versatility is the ability to attach or tag data to the image. The tags can include information such as optical density calibration, resolution, experimenter, date of capture, and any other data that the application software supports. TIFF images can be monochrome, 4-, 8-, or 16-bit gray-scale, or one of many color-image formats. Compression is optional, with LZW, RLE, and JPEG often supported (Russ, 1995). Since TIFF is supported by both Macintosh and PC computers, it is a good choice for multiple-platform environments. The versatility of TIFF can also be a weakness. Since there are many different tagging schemes and since not all programs support all possible compression and color schemes, it is sometimes not possible for one program to access the information in a TIFF file generated by a different program.

GIF (Graphics Interchange Format) is a file format that is widely encountered on the Internet due to its compactness and standardization. Its compactness is attributable to a mandatory modified LZW compression. Another feature of GIF is the use of a LUT to index the values in the image. One interesting ability of GIF is that it supports storing multiple images within a single file. This can offer some advantages for applications such as time-lapse image capture. A GIF image can contain no more than 256 individual color or gray levels and does not support intensity resolutions higher than 8 bit. In addition, since the image

is implemented as a LUT, it also is not a true gray-scale image. Due to these limitations and others, alternative formats such as PNG have been developed to replace GIF.

PICT is a file format and graphics metafile language (it contains commands that can be played back to recreate an image) designed for the Apple Macintosh. It can contain both bitmap images and vector-based objects such as polygons and fonts. It supports a ≤256-gray-level LUT, and monochrome images can be RLE compressed. Because it only offers a 256-gray-level LUT, it has the same weaknesses that GIF does with true gray-scale and high-intensity-resolution images. In addition, any vector objects in the image are difficult to translate on a PC since they are designed to be interpreted by Macintosh QuickDraw routines.

BMP is the native bitmap file format present on Windows-based PCs. It supports 2-, 16-, 256-, or 16-million-level images. With images of ≤256 gray levels, it implements a LUT, while the highest-resolution image is implemented directly. RLE compression is optional for 16- and 256-gray-level images. Since compression is prohibited on 16 million-gray-level images and there is no intermediate level supported beyond 256 levels, BMP is not a good choice for images with high-intensity resolution requirements.

ANALYSIS

Once the image has been captured, the data needs to be analyzed and distilled into information about the results of the electrophoresis experiment. Through the use of standards and experimenter input, this software-driven process can estimate mass and quantity of objects in an image and detect relationships between objects within one image and between similar images. The type of software used depends on the analysis to be performed. Images from single electrophonetic separations are examined by one-dimensional analysis software optimized for lane-based band detection. Images from two-dimensional electrophoresis are best handled by specific programs designed to detect spots and to assign two mobility values and a quantity value to the spot. After the initial characterization of bands and spots, comparisons are often made between bands or spots from different experiments through the use of database programs and matching algorithms.

Software for One-Dimensional Analysis

Lane positioning

For one-dimensional analysis, the first activity is to detect the lanes on the image. One of three different methods is commonly employed for this. For images with straight, well-defined lanes with a large number of bands, automatic lane-detection algorithms can quickly and accurately place the lanes. On images with very well-defined lanes, such as pseudoimages from finish-line type electrophoresis equipment, automated lane calling based on image position is possible. For images with "smiling," bent, or irregular lanes, manual positioning of the lanes is often the fastest and most accurate method of lane definition. Regardless of the method of identifying the lanes, the lane boundaries need to be carefully set for accurate quantitation and mass determinations. Lane widths should be wide enough so that the entire area of all bands in that lane are included, but they should not be so wide as to include bands from adjacent lanes. To accomplish this, curved or bent lanes might need to be used in order to follow the electrophoresis lane pattern. Lane length and position also must be adjusted as necessary so that all bands of interest are included. If mass determinations are necessary, the sample loading point should probably also be included in the lane or be the start of the lane. At this point, lines of equal mobility (often called R_f or iso-molecular-weight lines) are added to the image as necessary. These lines allow for correction of lane-to-lane deviations in the mobility of reference bands and generate more accurate measurements of mass. A similar form of correction is also possible for within-lane correction of mobilities. This correction is important for accurate detection and quantitation of closely spaced bands.

Band detection

Once the lanes have been defined, the bands present in each lane need to be detected. There are many methods for detecting bands. One method is to systematically scan the lane profile from one end to the other, identifying regions of local maxima as bands. Another common method is to use first- and second-order derivatives of the lane image or lane profile in order to find inflection points in the change of slope in pixel intensity values (Patton, 1995). Regardless of the method used, it is often necessary to alter the search parameters so that they perform reliably under a given experimental condition.

Typical search parameters include ones for detection sensitivity, smoothing, minimum interband gaps, and minimum or maximum band peak size. Smoothing reduces the number of bands detected due to noise in the image. A minimum interband gap is often used to avoid detection of false secondary bands on the shoulders of primary bands. Limitations on peak sizes, especially for within-lane comparison to the largest band's peak, can be a useful way to allow sensitive detection of bands in underloaded lanes without detecting false bands in overloaded lanes.

Band edges are often detected in addition to band peaks in order to further define bands or to quantitate band amounts. This can be accomplished by using local minima, derivatives of the lane profile, or fixed parameters such as image distances or a percentage of band peak height. The band edges can be applied as edges perpendicular to the long axis of the lane or as a contour of equal intensity circling the band. The perpendicular method is advantageous for bands with uneven distribution of material across the face of the band, while the latter method is better for "smiling" or misshapen bands.

Background subtraction

With nearly all electrophoresis procedures, the most informative images have a low level of signal intensity at each pixel that does not result from protein or nucleic acid. Instead, this background intensity is attributable to the gel medium, the visualization method, electronic noise, and other factors. Since this background tends to be nonuniformly distributed throughout the image, failure to subtract it can make band detection and quantitation less accurate. Many methods of background subtraction are possible. Sometimes it is possible to generate a second image under conditions that do not detect the protein or nucleic acid. The second image is then digitally subtracted from the data-containing image to remove the background. More often, background information must be obtained from a single image. If the background varies uniformly across the image, a line that crosses the variation can be defined at a point where no bands are present. The intensity values at each point on the line can be used as the background value for the pixels perpendicular to the line at that point. Commonly, background is also present as variations in intensity along the long axis within each lane. One simple method is to take the lowest point in the lane profile as the background. Another method is to use an average value of the edge of each band as the background for that band. More complicated methods such as valley-to-valley and rolling-disk use local minima points in the lane profile to define a variable background along the length of the lane. Because there can be many different causes and distributions of background, no single method of background determination can be recommended for all experiments.

Characterization

Once lanes and bands have been detected, it is possible to interpret the mobility of the nucleic acid or protein bands. Depending on the method of electrophoretic separation, information on mass (length or size), pI, or relative mobility (R_f) can be inferred from mobility information. The mobility is characterized using a standard curve with internal standards of known properties. The type of curve depends on several parameters. By definition, with R_f-based separation, a linear first-order curve is used since it represents the linear relationship between mobility and R_f. Similarly, pI and mobility are generally linear in isoelectric focusing separations. For separations based on size, a curve generated from mobility versus the log of the molecular weight provides a relatively good fit as measured by the correlation coefficient (R^2). Several other curves have been suggested for size-based separations, including modified hyperbolic curves and curves of mobility versus (molecular weight)$^{2/3}$ that have good correlation coefficients (Plikaytis et al., 1986). In some cases, no single curve equation can adequately represent the data, and methods of fitting smooth contiguous curves using only neighboring points, such as a Lagrange or spline fit (described in Hamming, 1973), are necessary. This is most common for size separations with a very large range of separation sizes and with nonlinear gradient gels. Care must be taken with multiple-curve techniques since they rely on only a few data points for any one part of the composite curve, and outlying data points can drastically affect the outcome.

For size and R_f determination, a uniform position must be found in each lane as a point from which to measure the mobility of each band. Many software analysis packages use the end of the lane as the measuring start point, so for them it is important to position each lane start point at an iso-molecular-weight or iso-R_f point. A convenient point is the well or sample-loading position since it is usually easily de-

tectable and at an equal mobility position in each lane. A consistent point on each band must also be chosen to measure mobility. A band's peak is easily defined in digital image analysis and is commonly used. Since peak positions are harder to detect visually than edges on silver halide images, the leading band edge is sometimes used when comparing digital results with silver halide–based results.

Once lanes and bands have been detected, it is also possible to quantify the amount or at least relative amount of nucleic acid or protein present in each band. The amount in a band is related to the sum total of the intensity values of each pixel subtracted by the background value for each pixel in a band. For absorptively detected bands, intensity values are converted to OD values. The total value that is calculated is equivalent to the volume of the band and can be directly compared to other bands that are within the linear range for the visualization method. If standards of known amounts are loaded onto the same gel, they can be used to generate a standard curve that converts band volume into standard units such as micrograms. For greatest accuracy, it is important to be able to generate multiple standard curves when using visualization methods, such as Coomassie blue staining, that are affected by band or spot composition.

Quantitation becomes more complicated when bands are not fully resolved. In this case, material from one band is contributing to the volume measurement of an adjacent band and vice versa. The simplest method for handling this is to partition into each band only the volume within its edges. Alternatively, a Gaussian curve can be fitted to each band and the volume contained within the curve used to estimate the amount of the band. Since most electrophoresis bands have a pronounced skew towards the leading edge of the band, modified Gaussian curves have also been used (Smith and Thomas, 1990). In either case, the curve-fitting process is calculation intensive and can significantly increase analysis times for images with many bands.

Software for Two-Dimensional Analysis

In two-dimensional analysis, the first-dimension separation is performed in a single column or lane and then a second separation is performed perpendicular to the first. The result after visualization is a rectangular image of up to 10,000 spots. The most common two-dimensional gel type is one in which protein is separated first by apparent pI and second by molecular weight, although two-dimensional separation of nucleic acids is also possible. While many of the concepts and analysis techniques used with one-dimensional gels are applicable to two-dimensional gels, the complex nature of most two-dimensional gels requires somewhat different methodology. For example, spots are more difficult to detect since they are not conveniently arranged in lanes and can vary more in shape and overlap than bands. In addition, two-dimensional experiments usually require some method of comparing between two images, whereas one-dimensional images usually contain all of the information from an experiment.

Spot detection

Probably the most difficult aspect of two-dimensional analysis is efficient and accurate spot detection. If it is incorrectly done, it can lead to hours of manual editing. Due to the complexity and computational intensity of some algorithms, the detection process itself can last hours on relatively fast desktop computers. One theoretically effective but computationally intense method is to treat the image as essentially a three-dimensional image with spots treated as hills and background as valleys. A large number of Gaussian curves are then combined to describe the topology of the image. Many other methods make use of a digital-imaging technique known as filtering. In essence, filtering is a way to weight the value of a pixel and its neighbors in order to generate a new value for a pixel. By passing a filter across an image pixel by pixel, a secondary image is generated. Filters can be designed for many tasks, including sharpening an image or removing high-frequency noise. Filters can also be generated to help detect spots by making images that are first and second derivatives of the original image. The derivative images indicate inflection points in the intensity pattern and can be used to detect spot centers and edges. In a different method, called thresholding, filters can be used to detect the edges of objects. Instead of looking for inflection points, threshold filters identify intensities above a set level or at ratios between central and edge pixels above a set value. Since the edges on two-dimensional spots tend to be diffuse, sharpening filters are sometimes used prior to the thresholding filter. In some cases, multiple techniques are used to detect spots (Glasbey and Horgan, 1994).

Unlike one-dimensional detection, detection on images of two-dimensional experiments usually requires secondary processing to get acceptable performance. One example of a secondary process is to discard spots with sizes below a set minimum or above a set maximum. Another is to analyze spots that are oval for possible splitting into two spots. Even after secondary processing, it is likely that a small amount of manual editing will be necessary. When manually editing an image, care must be taken to use as objective criteria as possible, especially if two or more images are to be matched and spot volumes compared.

Characterization

In a two-dimensional system, determination of protein or nucleic acid mobility is complicated by the fact that there are two mobilities to account for and that the second-dimension separation tends to make estimation of the separation which occurred in the first dimension more difficult. One method for dealing with this is to have a series of markers in the sample that, after both separations are completed, are evenly distributed within the gel and image. It is also possible to estimate separation characteristics from calibration points located at the periphery of the gel. For example, distance measurements can be used to pass calibration data from the first dimension separation, and standards can be separated at the ends of the gel to calibrate for mobility in the second dimension. Regardless of the method used, in many instances, a series of related images will be examined and similar spots in each image will be matched. When this occurs, it is possible to calibrate one image and then pass the calibration information via the matches to the related images.

Quantity determination is similar in many regards to that which occurs in one-dimensional analysis, but there are some differences. If spot edges are detected, a simple method of determining spot volume is to take the sum of the intensity value of each pixel in the spot reduced by a background intensity value. Multiple Gaussian curves can also be fitted to the spot to approximate the volume (Garrels, 1989). More difficult is attempting to compensate for a skewed distribution in a size-separating dimension while trying to use a regular Gaussian fit for a pI separation, such as is encountered with the most common form of two-dimensional protein separations. The distribution of background makes quantitation more difficult in two-dimensional gels. There is no lane-dependent component, so it is necessary to use other methods such as image stripes, finding local minimum values or using values derived from the spot edges to determine background values.

Matching

Matching is the process in which proteins or nucleic acids with similar separation properties are linked or clustered together. Matching can occur within one image or between multiple images as long as a frame of reference is established. Matching allows for comparisons between samples. It also makes annotation and data entry easier, since if one spot or band is matched to others and is characterized or annotated, this information is easily passed to all the other matches. An underlying assumption of matching is that objects with similar separation properties are actually similar. Care must be taken to confirm the identity of matched spots or bands by other methods on critical experiments.

A simple form of matching is to link bands or spots at similar positions on the gel images. This works well when separation and imaging conditions are uniform. This is very seldom the case, since slight differences in the electrophoresis, visualization, and imaging conditions across a gel and between gels generates incorrect matching with this method. Since bands on one-dimensional gels are relatively easy to calibrate for mobility, matching can occur along contours of equal mobility. This dramatically decreases but does not eliminate the variability in detecting similar bands. Much of the remaining variability can be attributed to calibration errors. This error can often be compensated for by allowing a small tolerance in mobility values in determining whether a band is matched or not. Because of the difficulties in calibrating mobility in two-dimensional gels, it is often more practical to use matched spots for calibrating mobility than vice versa. Spot matching between two two-dimensional images starts with finding a small number of landmark spots that are used as seeds for subsequent matches (Appel et al., 1991; Monardo et al., 1994). There are many methods for finding the landmarks in both images, including finding the highest-intensity spots, finding spots in unique clusters, and manual positioning. The most common procedure from this point is to derive a vector that describes the direction and extent of the path from one matched spot to the other when the two images are superimposed. The vector is used as the basis for finding more matches near the landmark matches. To allow

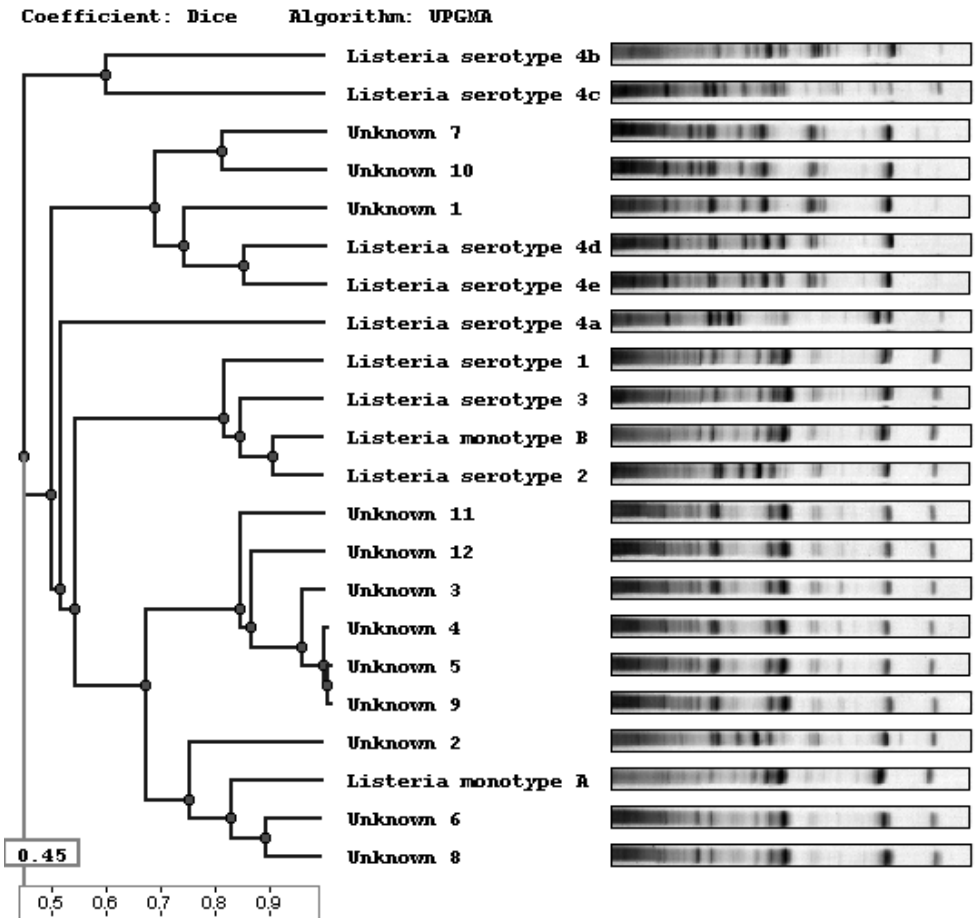

Figure 6.9.3 Example of a dendrogram generated from similarity data on band matching between lanes. DNA samples from 22 isolates of *Listeria* were subjected to Random Amplification of Polymorphic DNA (RAPD) analysis and the resulting electrophoresis image was analyzed with ImageMaster software (Amersham Pharmacia Biotech). Clustering was performed using the Dice coefficient with a tree structure based on the Unweighted Pair-Group Method using Arithmetic Averages (UPGMA). Similarity values between isolates can be determined by locating the node that connects the isolates and reading the value from the scale on the lower left edge of the dendrogram.

for error, the area within a small radius is searched extending from the end of the vector. Once another match is found, its vector is computed and used as the starting point for finding neighboring matches. From this progression, the entire gel is matched. If all vectors are displayed graphically when matching is complete, questionable matches can often be identified as vectors that are significantly different from neighboring vectors.

One specialized use of matching is as an estimator of the similarity and potential genetic relatedness of organisms. For example, on a one-dimensional gel image, a ratio of the matched to unmatched bands for each pairwise combination of lanes can be calculated. This ratio can be used as an indicator of similarity, with values near 1 indicating a pair of highly similar lanes, and values near zero indicating very dissimilar lanes. Assuming that the contents of the lanes are valid samples of the originating organism's genetic makeup, the information on lane similarity can be converted to estimates of genetic similarity. A convenient way to display this similarity data graphically is to generate a dendrogram with similar objects close to each other and less similar objects more distantly placed. An example of such a dendrogram is presented in Figure 6.9.3, where samples from *Listeria* isolates are arranged based on banding pattern.

Databases

In many cases, image analysis is not the last step in the process. The image and analysis data need to be archived in a searchable format. There may be a need to analyze the data from multiple experiments conducted at different sites or in laboratories around the world. Bioinformatic links to diverse data sources might be desired to help develop a unified understanding of the biology behind particular phenomena. When these situations arise, database programs can be utilized to store, link, and search image analysis results.

As the number of images that are captured and analyzed grows, it becomes increasingly more difficult to find particular information from the large number of files that are stored. Relatively simple databases can be used if the major requirement is to find previously analyzed images and associated data. Such databases often display a miniaturized version of each image to aid in visual scanning for the file as well as simple searching for image-specific information such as date of analysis, file name, or other information that was entered at the time of image capture. More powerful database products are also available that can perform complex searches on data generated during the analysis. For an example, a search on a two-dimensional database might include finding proteins exhibiting a specific expression profile and having a molecular weight >20 kDa with a pI between 3 and 5 or 8 and 10 with an amount <50 ng in a series of experiments conducted <1 year ago. Such searches can quickly target potentially interesting molecules for further analysis.

With the increasing ease of transferring data through the Internet as well as local- and wide-area networks, it has become practical to quickly find and examine data from distant locations. Of course, great care must be taken to ensure that similar experimental conditions are employed, as otherwise the results will be difficult to compare. In this manner it is sometimes possible to dramatically increase the sample size and statistical accuracy as well as the probability of detecting rare events. In addition, if one data set is more completely characterized, this extra information can be extracted and applied to the other data set. For example if there is a band in common in two databases and there is sequence information for it in one database, that sequence information can be added to the other database. Currently most public electrophoresis database sites are two-dimensional protein databases. A list with links to many of these Internet database sites can be found at *http://www-lmmb.ncifcrf.gov/EP/table2Ddatabases.html*.

With biological questions becoming more complicated and the answers to the questions often requiring information from a variety of sources, it is becoming increasingly important to be able to move easily between information sources. A relational type of database can help achieve this. Unlike a conventional database with a fixed arrangement of data, relational databases have links between related files that allow for easy movement from one file to another. Another approach to interconnecting electrophoresis data with data from other sources is to generate a series of hypertext links between data sets, similar to what occurs on the Internet. Selecting a specific link moves the search to the related network site and the related information. Regardless of the method, the end goal is similar. An example of what is possible: a researcher selects a protein spot on a two-dimensional gel image, which triggers accessing of related information on this protein. The protein sequence is accessed from mass spectroscopy analysis of the spot on a separate gel. The sequence of the gene and the cDNA that generated the protein is retrieved. The expression pattern of the gene in various tissues and conditions, as well as information on similar genes in other organisms, is incorporated. Citations and annotations to this are retrieved as well. All of this information is compiled automatically into an interactive report about the protein. From this report, the researcher can formulate a more refined hypothesis and plan the most appropriate experiments to test it.

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Provides an overview of image capture with particular emphasis on types of capture equipment.

INTERNET RESOURCES

rsb.info.nih.gov/nih-image

NIH Image is free software that provides basic image analysis tools for the Macintosh.

http://www.inforamp.net/~poynton/Poynton-color.html

Contains an excellent description of gamma correction in the Gamma FAQ.

http://www-Immb.ncifcrf.gov/EP/table2Ddatabases.html

A list of links to many two-dimensional databases that are available via the Internet.

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Two-Dimensional Blue Native Polyacrylamide Gel Electrophoresis

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ABSTRACT

Multiprotein complexes play crucial roles in nearly all cell biological processes. Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) is a powerful method to study these complexes. It is a native protein separation method that relies on the dye Coomassie blue to confer negative charge for separation. It has a higher resolution than gel filtration or sucrose density ultracentrifugation and can be used for protein complexes from 10 kDa to 10 MDa. If a second-dimension SDS-PAGE is applied (two-dimensional BN/SDS-PAGE), the size, subunit composition, and relative abundance of the different multiprotein complexes can be studied. In recent years, there has been a large increase in the number of publications where BN-PAGE was used to study protein-protein interactions. Here, we give detailed protocols for the separation of multiprotein complexes by two-dimensional BN/SDS-PAGE and for a related technique to determine the stoichiometry of these complexes. *Curr. Protoc. Cell Biol.* 38:6.10.1-6.10.21. © 2008 by John Wiley & Sons, Inc.

Keywords: multiprotein complex • native • gel electrophoresis • two-dimensional • Coomassie blue • protein-protein interaction

INTRODUCTION

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE; Basic Protocol 1) separates native proteins and protein complexes independently of their individual isoelectric points, with high-resolution capacity. This is done by conferring a negative charge on all proteins via the dye Coomassie blue. The separation depends mainly on the size of the protein complex. In combination with a second-dimension sodium dodecyl sulfate–PAGE (SDS-PAGE) in a perpendicular direction (Basic Protocol 2), it is an excellent choice to identify and characterize multiprotein complexes. It allows the determination of the size, subunit composition, and relative abundance of different multiprotein complexes from total cell and tissue homogenates, as well as from purified material. In addition, it is used for a one-step preparative purification of protein complexes.

One critical step in performing high-quality BN-PAGE is the preparation of the sample, which has to be devoid of potassium and divalent cations. The most useful source for protein complexes is a cellular lysate or tissue extract. Preparation of a cellular lysate suitable for separation by BN gels is done by dialysis to remove any cations and metabolites (see Support Protocol). A detailed protocol for pouring and running of the BN gradient gels (see Basic Protocol 1) and denaturation of the separated proteins followed by separation using a second dimension SDS-PAGE (see Basic Protocol 2) is given. Visualization of the resulting two-dimensional gel can be done according to standard protocols, including general protein stains (*UNIT 6.6*) and immunoblotting, often referred to as western blotting (*UNIT 6.2*).

A second dimension is not always required. Proteins can be detected after BN-PAGE by such standard procedures as general protein stains or transfer of the proteins to a

Current Protocols in Cell Biology 6.10.1-6.10.21, March 2008
Published online March 2008 in Wiley Interscience (www.interscience.wiley.com).
DOI: 10.1002/0471143030.cb0610s38
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membrane under denaturing (see Alternate Protocol 1) or native (see Alternate Protocol 2) conditions followed by immunodetection. Using these protocols, there is a special requirement for the antibodies used to detect the proteins of interest, because Coomassie blue interferes with fluorescence-based visualization methods and because native proteins are often detected by different antibodies than those that detect denatured proteins.

A powerful method to determine the stoichiometry of multiprotein complexes is the Native Antibody-based MObility Shift (NAMOS) assay (see Basic Protocol 3). It is based on one-dimensional BN-PAGE and uses the fact that proteins migrate more slowly during the electrophoresis when an anti-subunit antibody is bound.

NOTE: High-purity water (e.g., Milli-Q or distilled water) should be used for all solutions. For cautions relating to electricity and electrophoresis, see Safety Considerations in the introduction to *UNIT 6.1*.

NOTE: Wear powder-free gloves throughout the procedure and work on ice or at 4°C whenever native proteins/protein complexes are present.

CAUTION: Acrylamide is hazardous; see *APPENDIX 2A* for guidelines on handling, storage, and disposal.

BASIC PROTOCOL 1

FIRST-DIMENSION BLUE NATIVE ELECTROPHORESIS

In this protocol, the pouring and running of vertical slab blue native (BN) gels is described. BN gels are gradient gels of low acrylamide percentage and strength. Thus, pouring and handling of the gels is not trivial. The gradient has to be even, in order to prevent any step that would result in the erroneous accumulation of proteins at a particular height of the gel. The BN gel solution with the higher acrylamide/bisacrylamide concentration is heavier than the low-percentage gel because of its high glycerol content. This density difference aids in establishment of a uniform gradient inside the glass plates. The same gel equipment that is used for normal SDS-PAGE can be used, but one has to be certain that no traces of SDS are present. To ensure absence of any detergent, the BN equipment should not be used for SDS-PAGE. BN gels are poured at room temperature and are cooled to 4°C before samples are loaded in the cold room. Alternatively, gel apparatuses that allow cooling can be used. Precast BN gels have recently been made commercially available, but in the author's experience, these are not as good as self-made gels.

It is strongly recommended to use multicasting equipment to pour several gels at once. This avoids steps of the gradient, saves time, and ensures best reproducibility for critical comparisons of multiple samples. Casting of multiple gradient gels is described in *UNIT 6.1*, Support Protocols 2 and 3, and Figures 6.1.3 and 6.1.4. Using these protocols, BN gels can be prepared using BN-specific solutions.

Materials

Low-percentage BN separating gel solution (see recipe)
High-percentage BN separating gel solution (see recipe)
Isobutyl alcohol
3× BN gel buffer (see recipe)
BN stacking gel solution (see recipe)
100× pervanadate solution (optional, if phosphorylation must be preserved; see recipe)
Sample: dialyzed cell lysate (Support Protocol), tissue homogenate, purified protein complex
Marker mixes 1 and 2 (see recipe)
BN anode buffer (see recipe)

BN cathode buffer (with 0.02% w/v Coomassie blue; see recipe)
BN cathode buffer (with low, 0.002% w/v Coomassie; see recipe)

Gel electrophoresis apparatus (see *UNIT 6.1*)
Gradient mixer (self-made or, e.g., from BioRad; Fig. 6.1.2)
Peristaltic pump (Fig. 6.1.2)
Power supply

Additional reagents and equipment for polyacrylamide gel electrophoresis (*UNIT 6.1*) and protein staining in gels (*UNIT 6.6*)

Set up the apparatus

1. Wash glass plates and pouring devices extensively with water.

   Do not use equipment that has been previously used for SDS-PAGE. No traces of detergent should be present on the glass plates

2. Assemble the glass plate sandwich of an electrophoresis apparatus.

   UNIT 6.1 describes these procedures in detail.

Prepare the gradient

3. Set up the equipment to pour gradient gels as shown in Figure 6.1.2 (*UNIT 6.1*).

   Gels will be poured at room temperature. For reproducibility, and to make the pouring more effective, it is recommended to pour at least 10 gels at once using multicasting equipment (e.g., BioRad).

4. Close the valves and place a magnetic stir bar into the mixing chamber of the gradient maker. Prepare low- and high-percentage BN separating gel solutions (see Reagents and Solution). Adjust the acrylamide/bisacrylamide concentration of the high-percentage BN separating gel solution according to your needs (Table 6.10.1). Add APS and TEMED only immediately before use.

   The volumes of the two solutions combined should be exactly equal to the volume required to fill the glass plate sandwich to the required height.

   When using multicasting equipment, the dead volume of the apparatus has to be determined empirically and added to the required volume of the gel solutions.

5. Pour the low-percentage (light) BN separating gel solution into the reservoir chambers and the high-percentage (heavy) BN separating gel solution into the mixing chamber of the gradient mixer (Fig. 6.1.2).

6. Open the interconnecting valve and force out the air bubble inside the connecting channel by pressing over the right cylinder with your thumb.

   CAUTION: *Make sure to wear gloves, in order to avoid contact with acrylamide.*

Cast the Blue Native separating gels

7. Switch on the peristaltic pump, open the outlet valve, and allow the gel to slowly enter between the glass plates. Ensure that the needle at the end of the Tygon tubing is always above the height of the liquid, so that the gradient is not disturbed.

   When using multicasting equipment, the gel solutions enter between the glass plates from the bottom. In this case, the low-percentage BN separating gel solution has to be placed into the mixing chamber and the high-percentage one into the reservoir chamber (Fig. 6.1.3). Figure 6.1.2 shows a pipet tip at the end of the Tygon tubing, but a needle may be more appropriate since a pipet tip might be too wide to fit between the glass plates.

8. Prepare water-saturated isobutyl alcohol by shaking isobutyl alcohol and water in a glass bottle. Using a Pasteur pipet, overlay the separating gel with water-saturated isobutyl alcohol (upper alcoholic phase from the mix) by gently layering the alcohol

Table 6.10.1 Gel Solutions for BN-PAGE[a]

	Low% BN separating gel solution	High% BN separating gel solution					Stacking gel solution
	4%	7%	10%	12%	16%	18%	3.2%
40% acrylamide/ bisacrylamide mix[b] (ml)	3.00	5.25	7.50	9.00	12.00	13.50	1.00
3× BN gel buffer[b] (ml)	10.00	10.00	10.00	10.00	10.00	10.00	4.17
dH_2O (ml)	17.00	—	—	—	—	—	7.33
70% (v/v) glycerol (ml)	—	14.75	12.50	11.00	8.00	6.50	—
10% ammonium persulfate (µl)	108	84	84	84	84	84	167
TEMED (µl)	11	8	8	8	8	8	17

[a]Numbers in the body of the table are milliliters of stock solution, except 10% APS and TEMED which are microliters and should be added only when the low- and high percentage BN gel solutions are already in the chambers of the mixing apparatus.
[b]See recipe in Reagents and Solutions.

against the edge of one and then the other of the spacers to produce a smooth surface.

In the multicasting equipment, all gels must be overlaid with the same volume of water-saturated isobutyl alcohol.

9. Clean pouring apparatus with water (do not use detergent).

10. Allow the gel to polymerize for at least 30 min at room temperature. Remove the isobutyl alcohol, wash twice with water, and overlay with 1× BN gel buffer. Store at 4°C (stable at least 1 year).

Pour the stacking gel

11. Just before use, pour the stacking gel using an appropriate comb and the BN stacking gel solution (see Reagents and Solutions).

Due to the low percentage of acrylamide/bisacrylamide, the comb might be difficult to remove without damaging the gel. Try to move the comb perpendicular to the plane of the glass plates while removing it to allow air to enter. Alternatively, the percentage of acrylamide/bisacrylamide can be increased to 3.5%.

Load the samples and run the Blue Native gels

All steps should be performed at 4°C (e.g., in a cold room).

12. Add 100× pervanadate solution to the dialysed lysate to a concentration of 1×, if phosphorylation needs to be preserved.

13. Mount the gel in the electrophoresis apparatus and load 5 to 30 µl dialysed cell lysate (prepared as in Support Protocol) in the dry wells of the mounted gel.

As control, an aliquot of the sample can be boiled with 1% SDS to destroy all multiprotein complexes. Leave one lane free between this control and the non-SDS samples.

14. Load 10 µl marker mix 1 and 10 µl marker mix 2 in two adjacent wells.

Only ferritin is seen during the electrophoresis, due to its brown color.

Alternatively, commercially available high-molecular-weight markers can be used (e.g., from Invitrogen or GE Healthcare).

15. Carefully overlay the samples in each well with the BN cathode buffer. Fill the upper/inner chamber with BN cathode buffer (containing 0.02% w/v Coomassie blue as described in Reagents and Solutions) and the lower/outer chamber with BN anode buffer.

16. Connect electrodes. If a small gel has been prepared (e.g., using BioRad Protean II or III), run at 100 V; if a large gel has been prepared (e.g., using BioRad Protean II xi), run at 150 V. Continue electrophoresis at the appropriate abovementioned voltage until the sample has entered the separating gel. At that point, increase voltage to 180 V for small gel or 400 V for large gel.

    Coomassie blue comes into contact with the samples inside the wells during the electrophoresis.

17. Remove the BN cathode buffer (especially from within the wells) after two-thirds of the gel run. Fill the upper/inner chamber with BN cathode buffer (low Coomassie blue) that contains only 0.002% (w/v) Coomassie blue. Continue electrophoresis at 180 V for small gel or 400 V for large gel.

    This procedure ensures that the individual lanes can be identified after the gel is run and can be omitted if a second-dimension gel is not to be applied (see, e.g., Alternate Protocols 1 and 2), or if precipitated material is visible between the stacking and separating gel.

18. Turn off power supply and remove the electrodes once the dye front has reached the bottom of the gel.

Extrude and store Blue Native gels

19. Remove the glass plates, with the gel in-between, from the electrophoresis apparatus.

20. Open the glass plates by lifting the smaller one and keeping the gel attached to the bigger, bottom plate. Remove the stacking gel with the smaller glass plate.

21. Cut away the lanes where the marker mixes were loaded from the rest of the gel using the smaller glass plate. Visualize the marker proteins by standard Coomassie blue or silver staining (*UNIT 6.6*).

    For some purposes, the ferritin marker (440 and 880 kDa), which is visible without staining, is sufficient. When assigning the positions of the marker, take into consideration that the gel might shrink upon staining.

    Alternatively, after the first-dimension BN-PAGE, proteins can be stained directly with silver or Coomassie brilliant blue (UNIT 6.6), or detected by immunoblotting (western blotting; Alternate Protocols 1 and 2). Optionally, stained spots can be cut out and the proteins identified by mass spectroscopy.

22. Stamp out each individual lane with the smaller glass plate. Either immediately run the second dimension SDS-PAGE (Basic Protocol 2), or freeze each lane wrapped in aluminum foil at −20°C (stable at least 1 year).

    Do not bend a frozen gel piece, since it breaks easily. Alternatively, the first-dimension BN gel can be transferred to a membrane followed by immunoblotting to visualize the proteins of interest (Alternate Protocols 1 and 2).

PREPARATION OF CELL LYSATES FOR BLUE NATIVE GEL ELECTROPHORESIS

SUPPORT PROTOCOL

Blue Native (BN) gel electrophoresis is suitable for the separation of pure proteins as well as complex protein mixtures such as subcellular fractions or cell lysates. Sample preparation is one of the critical steps in performing high-quality BN gels. First, the proteins have to be present in soluble and native form. Thus, the choice of detergent is important and one should start BN experiments by testing several nonionic detergents

for the protein/protein complex of interest (see Commentary). The detergent should be effective enough to extract the proteins from cellular membranes, but at the same time mild enough to keep multiprotein complexes intact. The most commonly used detergents are given in Reagents and Solutions. The proteins must be eluted in native form, if the purification procedure includes binding to a matrix, as e.g., in immuno- or affinity purifications (often referred to as immunoprecipitation; see *UNIT 7.2*). Second, the sample has to be prepared without potassium or divalent cations, since Coomassie blue and Coomassie blue–bound proteins precipitate with those ions and consequently do not enter the gel. Hence, cations that interact with Coomassie blue have to be removed and substituted by 6-aminohexanoic acid, in order to maintain a certain ionic strength of the solution, which is necessary for the solubility and stabilization of many protein complexes. Sodium ions are tolerated to a maximum concentration of 50 mM. Third, samples have to be loaded with detergent on BN gels. Otherwise, proteins aggregate during the stacking step of the electrophoresis.

Membrane and organelle fractions lysed in BN lysis buffer can be directly applied to BN gels. Cellular lysates have to be dialysed against BN dialysis buffer, in order to remove small cations and metabolites. Proteins bound to a matrix are washed and eluted in BN dialysis buffer.

This protocol describes the preparation of cell lysates. Cells are washed and lysed in any lysis buffer, although we recommend using the BN lysis buffer. After removal of insoluble material, the lysate is dialysed against BN dialysis buffer. In this protocol, a self-made dialysis setup utilizing dialysis membranes is described. Alternative desalting methods can be applied as well.

Materials

Cell culture dish (10 to 15 cm) containing cells of interest (80% confluent)
Phosphate-buffered saline (PBS; see recipe), ice cold
BN lysis buffer (see recipe)
BN dialysis buffer (see recipe)

Cell scrapers
10- to 50-ml centrifuge tubes
Refrigerated cell culture centrifuge (with adaptor cavities for microcentrifuge tubes in rotor accommodating 50-ml tube)
Dialysis membranes, MWCO 10 to 50 kDa (boiled and kept at 4°C in 0.001 M EDTA)
1-ml reaction tubes (e.g., microcentrifuge tubes)
Beaker (100-ml to 1-liter, depending on sample size)

Harvest and wash the cells

1. Place cell culture dish containing cells on ice.
2. For adherent cells, wash cells three times, each time with 2 to 10 ml ice-cold 1× PBS.

   Cells grown in suspension are washed three times with ice-cold 1× PBS by repeatedly pelleting the cells by centrifugation for 5 min at 350 × g, 4°C.

   The volume of PBS used for one washing step should be equal to the volume of medium in which the cells were cultivated.
3. Add 2 to 10 ml (i.e., volume in which cells were cultivated) of ice-cold 1× PBS to the dish, remove the cells using a cell scraper, and transfer the cell suspension to a 10- to 50-ml centrifuge tube.

   This step is omitted for cells grown in suspension.

4. Collect cells by centrifugation for 5 min at 350 × *g*, 4°C, and remove supernatant.
5. Resuspend cells in 1 ml cold 1× PBS, transfer cell suspension into a small reaction tube (e.g., microcentrifuge tube), and pellet cells by centrifugation for 5 min at 350 × *g*, 4°C.
6. Remove and discard the supernatant using a pipet.
7. Either move on to step 8 or freeze the cell pellet at −20°C.

   Frozen cells can be stored at −20°C for at least 6 months.

Prepare the cell lysates

8. Resuspend the cell pellet in ice-cold BN lysis buffer (or other lysis buffer that has been used successfully to extract your multiprotein complex of interest).

   The concentration of cells per volume lysis buffer depends on the amount of protein that should be loaded on the BN gel. As an approximation, use 10^6 cells per 250 to 500 µl lysis buffer.

9. Incubate on ice for 15 min to allow cell lysis.

   The lysis time can be prolonged, if it is convenient—e.g., in the case where many different samples are processed and some of them have to wait for the last ones.

10. Microcentrifuge 15 min at 13,000 × *g*, 4°C, to remove insoluble material.

Prepare to dialyze the lysate

11. Melt a hole in the cap of a microcentrifuge tube using the large-diameter end of a hot Pasteur pipet. Chill the tube on ice.
12. Transfer the supernatant from step 10 into the chilled tube from step 11.
13. Place a dialysis membrane over the opened tube and close the cap.

    Make sure that there are no folds or tears in the dialysis membrane.

14. Seal the cap at the edges with Parafilm.

    Make sure that the hole in the tube is not covered by the Parafilm.

15. Invert the tube and centrifuge upside-down at the lowest speed possible in the adaptor cavity for 50-ml tubes in a cell culture centrifuge for 10 sec at 4°C.
16. Prepare a 100-ml beaker with ice-cold BN dialysis buffer and a magnetic stirrer. Use at least 10 ml dialysis buffer per 100 µl sample.
17. Fix the tube upside down inside the beaker and remove air bubbles from the hole beneath the cap.

    Make sure that the dialysis membrane is not damaged.

Dialyze lysate

18. Switch on the magnetic stirrer and dialyze for 6 hr or overnight in a cold room.

    Make sure that stirring is not creating air bubbles at the dialysis membrane.

19. Collect the dialysed cell lysate in a new chilled microcentrifuge tube and analyze by BN-PAGE (Basic Protocol 1).

    If the sample will be subjected to two-dimensional analysis, reserve one-third of the sample to serve as a control in the second-dimension analysis.

    Freezing and thawing of cell lysates might lead to the aggregation of proteins. Therefore, the cell lysate should be separated immediately by BN-PAGE. For some proteins, freezing might be possible and has to be determined empirically.

BASIC PROTOCOL 2

SECOND-DIMENSION DENATURING ELECTROPHORESIS

A denaturing second-dimension gel is suitable to identify and characterize multiprotein complexes. It is recommended to use vertical denaturing (SDS) discontinuous gel electrophoresis (Laemlli method of SDS-PAGE; described in detail in *UNIT 6.1*, Basic Protocol 1). These gels can be linear or gradient slab gels run under reducing or nonreducing conditions. The only exception is that loading of the first-dimension BN gel strip requires a broad, flat well. The BN gel strip has to be fit between the glass plates of the second gel. The easiest way is to slightly increase the thickness of the second dimension compared to the first dimension. If this is done by putting cellophane tape on the spacers of the second dimension gel, the BN gel strip can be inserted without breaking and still sit firmly enough to prevent any movement during the electrophoresis. Alternatively, procedures as described in *UNIT 6.4* can be employed.

This protocol describes all the specific steps required for successfully casting and running the second-dimension SDS-PAGE, with extensive reference to *UNIT 6.1*. Typical expected results are depicted in Figure 6.10.1.

Materials

2× SDS sample buffer (*UNIT 6.1*)
1× SDS electrophoresis buffer (*UNIT 6.1*)
Lane from a BN-PAGE gel (Basic Protocol 1)

Thin adhesive tape (e.g., tesa tape; *http://www.tesatape.com*)
Platform shaker

Additional reagents and equipment for SDS-PAGE (*UNIT 6.1*) and two-dimensional gel electrophoresis (*UNIT 6.4*)

Cast the second-dimension SDS gels

1. Take spacers of the same thickness as the ones that were used for the first dimension (see Basic Protocol 1). Wrap thin adhesive tape once around them, in order to increase their thickness slightly.

2. Assemble the glass-plate sandwich using these spacers.

   A second-dimension gel that is slightly thicker than the first-dimension gel allows fitting in the first-dimension gel slice without problems, but still fixing it between the glass plates so that it cannot move.

3. Pour the separating SDS-PAGE gel as described in *UNIT 6.1*.

   For everyday purposes, degassing of the gel solution is not necessary. It is recommended to use multicasting equipment to pour several SDS gels at once. It saves time and ensures best reproducibility for comparisons of multiple samples. Casting of multiple gradient gels is described in UNIT 6.1.

4. Pour the stacking gel (*UNIT 6.1*) using combs that contain one large pocket that is wide enough to accommodate the first-dimension gel slice and one or two additional wells for marker and control samples (*UNIT 6.4*).

   After removing the comb, clean the large pocket of polymerized gel remnants using a spacer that has not been enlarged with a layer of tape.

Load the Blue Native gel strips onto the second-dimension SDS gels

5. Retrieve the first-dimension BN gel strips from storage. Incubate them for 10 min at room temperature in 2× SDS sample buffer by shaking in a small tray on a platform shaker.

   Make sure to work under a hood if working with a reducing sample buffer containing 2-mercaptoethanol.

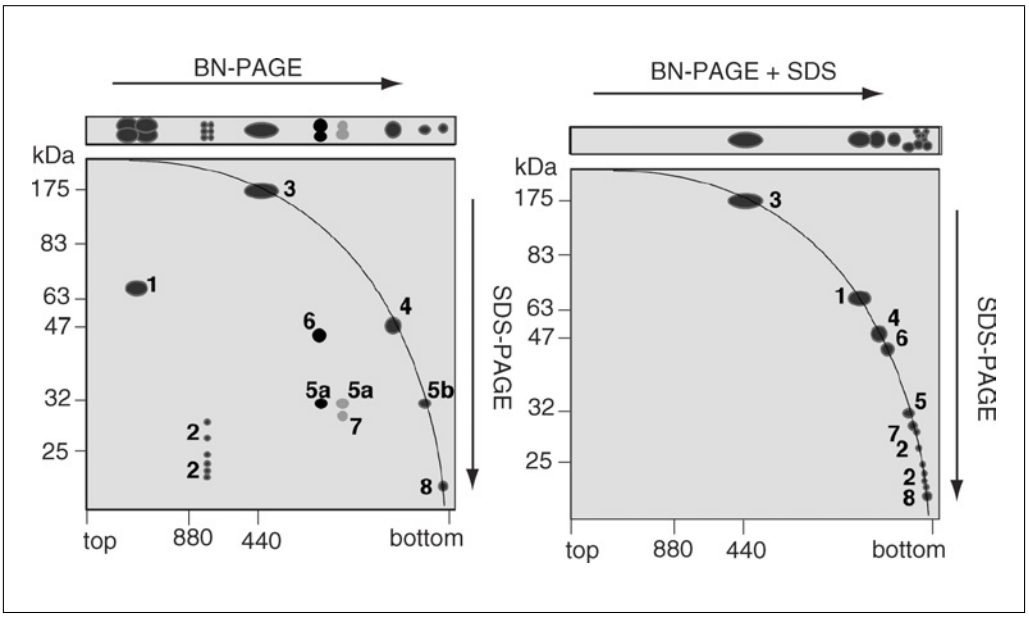

Figure 6.10.1 Two-dimensional BN/SDS-PAGE of cellular lysates. In this hypothetical experiment, total cellular lysates were prepared and dialyzed (Support Protocol). In (**A**), proteins were separated by BN-PAGE (Basic Protocol 1) and a subsequent second-dimensional SDS-PAGE (Basic Protocol 2). Visualization of the protein was done with silver staining. Monomeric proteins (#3, #4, and #5b) were localized to a hyperbolic-shaped diagonal, indicating that they had the same size in the first and in the second dimension. In contrast, proteins that were present in the same multiprotein complex were found as individual spots aligned in vertical columns. Complex #1 was a homotetrameric protein complex. Complex #2 was composed of six proteins of small individual sizes. Proteins #6 and #5 as well as #7 and #5 formed dimeric complexes. In addition, protein #5 was found as a monomer (#5b). Thus, protein #5 existed in three different forms. From the intensity of the spots, one could deduce that complex #6:#5 was more abundant than complex #7:#5. In (**B**), the dialysed lysate was boiled with 1% SDS before separation by BN-PAGE and SDS-PAGE. SDS destroys all multiprotein complexes; thus, the hyperbolic-shaped diagonal can identified. A similar wet experiment is described in Camacho-Carvajal et al. (2004).

6. Heat the strips with the sample buffer in a microwave oven at medium power until the solution boils. Let the gel strips shake for another 10 min at room temperature while cooling down.

   Heating the proteins in the presence of SDS leads to their unfolding and to the disruption of multiprotein complexes.

7. Fill the large pocket of the second-dimension gel with 1× SDS electrophoresis buffer, removing all air bubbles. Incline the gel with the large glass plate at the bottom. Let the first-dimension gel slice enter between the glass plates. Avoid any air bubbles and push carefully with a spacer without added tape.

8. Place the gel into the electrophoresis apparatus and load the markers and control samples into the small wells.

   It is recommended to keep one-third of the sample to be separated by the first dimension BN-PAGE (Support Protocol, step 19), boil it in 1× SDS electrophoresis buffer, and use it as a control sample in the second dimension.

9. Overlay the first-dimension gel strip with a 3-mm layer of 2× SDS sample buffer.

Run and analyze the second-dimension SDS gel

10. Run and disassemble the gel as described in *UNIT 6.1* (Basic Protocol 1).

    The dye front contains not only the bromphenol blue of the SDS sample buffer but also the Coomassie blue from the first dimension. The dye front of the first dimension will be visible as a dark blue spot at the bottom of the second dimension.

11. Visualize proteins using any method available.

The most commonly used methods are transfer to a membrane followed by immunodetection (western blotting; UNIT 6.2) or staining with silver or Coomassie brilliant blue (UNIT 6.6). Optionally, stained spots can be cut out and the protein identified by mass spectroscopy.

ALTERNATE PROTOCOL 1

DENATURING TRANSFER OF THE PROTEINS FROM THE FIRST-DIMENSION BN-PAGE GEL TO A MEMBRANE FOR IMMUNOBLOTTING

Protein complexes separated by BN gels can be visualized by immunoblotting (also referred to as western blotting) without a second-dimension separation. If a cell lysate is analyzed, a very specific antibody is necessary that does not cross-react with other proteins. It is recommended to first test the quality of the antibody by second-dimension BN/SDS-PAGE (see Commentary). In general, the same antibodies that are used for immunodetection after SDS-PAGE are potentially suitable.

In this protocol, the proteins/multiprotein complexes are first denatured within the BN gel by boiling in SDS. Subsequent steps, including the transfer to a membrane and the immunoblotting procedure, are similar to the standard protocol used after SDS-PAGE (*UNIT 6.2*). If PVDF membranes are used, the bound Coomassie blue can partially be removed. This is not the case for nitrocellulose membranes that can be used to immobilize the proteins after BN-PAGE as well.

Materials

First-dimension BN-PAGE gel (Basic Protocol 1)
Phosphate buffered saline (PBS; see recipe for 10×) containing 1% (w/v) SDS
Denaturing BN transfer buffer (see recipe)
Destaining solution (see recipe)

Platform shaker
PVDF (polyvinylidene fluoride) membrane (see *UNIT 6.2*)

Additional reagents and equipment for immunoblotting (*UNIT 6.2*)

1. Soak the first-dimension BN gel in 1× PBS with 1% SDS for 15 min by shaking in a small tray on a platform shaker.
2. Heat in a microwave at medium power until the solution boils. Let the gel shake for another 10 min at room temperature.
3. Transfer the denatured proteins to a PVDF membrane by wet or semi-dry blotting (*UNIT 6.2*). Use the denaturing BN transfer buffer, since it includes SDS.

   A protocol for wet transfer is described in UNIT 6.2 (Basic Protocol 1, including Fig. 6.2.1). Semi-dry transfer is described in UNIT 6.2 (Alternate Protocol 1, including Fig. 6.2.2).

   Before transfer, mark the ferritin marker with a pen on the membrane, since it is difficult to see afterwards.

4. *Optional:* Partially remove the transferred Coomassie blue from the PVDF membranes by incubation in destaining solution for 30 min.

   Immunodetection can also be performed without the destaining step. Coomassie blue cannot be removed completely and shows strong fluorescence at several wavelengths. Therefore, detection with fluorescently labeled antibodies cannot be done. In this case a second-dimension SDS-PAGE has to be used for immunoanalysis (Basic Protocol 2).

5. Block the membrane and immunodetect under standard conditions (*UNIT 6.2*).

NATIVE TRANSFER OF THE PROTEINS FROM THE FIRST-DIMENSION BN-PAGE GEL TO A MEMBRANE FOR IMMUNOBLOTTING

ALTERNATE PROTOCOL 2

Proteins are separated by BN-PAGE under native conditions. Transfer to a membrane and visualization by immunoblotting can also be performed under native conditions. If a cell lysate is analyzed, a very specific antibody that does not cross-react with other proteins is necessary. The antibodies that are used for immunodetection after SDS-PAGE might not be suitable, since they recognize unfolded proteins. Antibodies that recognize folded proteins and protein complexes, as used for immunoisolation (e.g., immunoprecipitation), flow cytometry, or immunofluorescence methods, should be tested for the application in this protocol.

In this protocol, the proteins/multiprotein complexes are transferred to a membrane in their native form. The following immunoblotting procedure is similar to the standard protocol used after SDS-PAGE (*UNIT 6.2*).

Materials

First-dimension BN-PAGE gel (Basic Protocol 1)
Native BN transfer buffer (see recipe)
Destaining solution (see recipe)
PVDF membrane (see *UNIT 6.2*)

Additional reagents and equipment for immunoblotting (*UNIT 6.2*)

1. Briefly rinse the first-dimension BN gel in native BN transfer buffer.
2. Transfer the native proteins to a PVDF membrane by semi-dry or wet blotting (*UNIT 6.2*). Use the native BN transfer buffer, since it does not contain SDS.

   Conditions for transfer are the same as those used for SDS-PAGE gels (UNIT 6.2).

   Before transfer, mark the ferritin marker with a pen on the membrane, since it is difficult to see afterwards.

3. *Optional:* Partially remove the transferred Coomassie blue from the PVDF membranes by incubation in destaining solution for 30 min.

   Immunodetection can also be performed without the destaining step. Coomassie blue cannot be removed completely and shows strong fluorescence at several wavelengths. Therefore, detection with fluorescently labeled antibodies cannot be accomplished. In this case a second-dimension SDS-PAGE has to be done (Basic Protocol 2).

4. Block the membrane and immunodetect under standard conditions (*UNIT 6.2*).

NATIVE ANTIBODY-BASED MOBILITY SHIFT (NAMOS) ASSAY

BASIC PROTOCOL 3

This protocol is a variant of Basic Protocol 1 and describes a method to determine the stoichiometry of multiprotein complexes based on BN-PAGE. It makes use of the fact that the proteins and protein complexes are separated in their native state. Antibodies that recognize a certain subunit of a protein complex are added to the sample before separation by BN-PAGE. This allows the formation of super-complexes comprising the protein complex and the antibody. Since the migration distance in BN-PAGE depends on the size of a protein complex, it directly reflects the number of bound antibodies and thus the copy number of the subunit they bind to. This method is called the Native Antibody-based MObility Shift (NAMOS) assay. With the NAMOS assay, it is even possible to determine the stoichiometry of a protein complex that is present in low amounts within a total cell lysate. Due to the large amount of added antibody, detection of the protein complex of interest has to be done by immunoblotting as described in Alternate Protocols 1 and 2, and *UNIT 6.2*.

Before performing the NAMOS assay, one has to set up the sample preparation (Support Protocol 1) and immunodetection (Alternate Protocols 1 and 2) conditions. It is required that the multiprotein complex of interest be separated as a single, clearly visible complex after BN-PAGE.

Typical expected results are displayed in Figure 6.10.2.

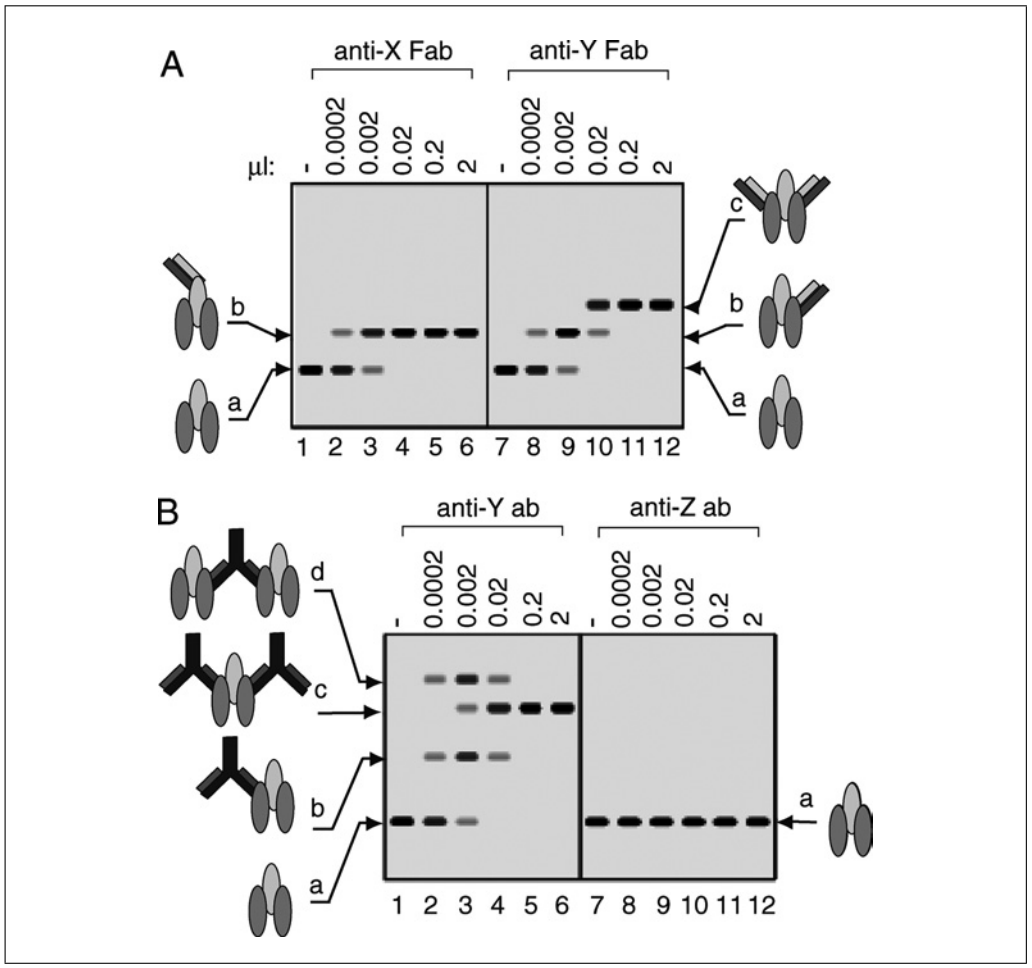

Figure 6.10.2 The Native Antibody-Based Mobility Shift (NAMOS) Assay. In this hypothetical experiment, the protein complex of interest comprises one X and two Y subunits (XY_2). Its stoichiometry is to be determined by the NAMOS assay (Basic Protocol 3). Cellular lysates containing complex XY_2 were separated by BN-PAGE and visualized by anti-XY_2 immunoblotting. Indeed, XY_2 was one defined complex (lanes 1 and 7, band a). In (**A**), increasing amounts of an anti-X Fab fragment were incubated with the lysate before separation. Consequently, a new band (band b) appeared with a concomitant loss of band a (lanes 2 to 6). Band b corresponded to a Fab-XY_2 complex, indicating that XY_2 contained one binding site for the anti-X Fab fragment and, thus, one copy of protein X. An anti-Y Fab fragment produced two bands (lanes 8 to 12, bands b and c), demonstrating that XY_2 had two copies of protein Y. In (**B**) complete antibodies were used for the NAMOS assay. In contrast to the Fab fragment, an antibody is able to simultaneously bind to two XY_2 complexes. Using anti-Y, one can see that these "super"-complexes (band d) disappeared with increasing concentrations of the antibody (lanes 2 to 5). At saturating concentrations, all XY_2 complexes bound to two antibodies (lanes 5 and 6, band c), indicative of two copies of protein Y in the complex. Thus, complete antibodies also allow the determination of stoichiometry. As a control, an irrelevant anti-Z antibody did not produce any shift of complex XY_2 (lanes 7 to 12). A similar wet experiment and possible difficulties in performing the NAMOS assay are described in Swamy et al. (2007).

Materials

Monoclonal antibodies that react with the proteins of interest
BN dialysis buffer (see recipe)
Sample for BN-PAGE
1-ml reaction tubes (e.g., microcentrifuge tubes)

Additional reagents and equipment for casting and running BN-PAGE gels (see Basic Protocol 1) and analysis of Blue Native gels by immunoblotting (Alternate Protocol 1 or 2)

Prepare the samples and load the Blue Native gel

1. Prepare a dilution series of the monoclonal antibodies of interest using the BN dialysis buffer containing the same detergent as the sample. Start with ~1 μg of antibody in 2 μl in the first reaction tube, then dilute 1:10, 1:100, 1:1,000, and 1:10,000 in another four tubes. Place the tubes on ice.

   Antibodies are mostly dissolved in PBS and, thus, the nondiluted antibody will be present with sodium chloride. If only 2 μl are used, the resulting salt concentration is tolerated by BN-PAGE. If, however, more than 2 μl have to be used, it is recommended to dialyze the antibody against BN dialysis buffer without any detergent (Support Protocol). Use fresh antibodies that have not been frozen and thawed too often, in order to avoid the presence of antibody aggregates. If possible, use Fab fragments of the antibodies.

2. Use any sample as described in the Support Protocol, e.g., a dialysed lysate or purified proteins. Add 10 to 20 μl of sample to each antibody-containing tube, mix, and incubate 20 min on ice.

3. Cast and pour the BN gel as in Basic Protocol 1. Use an acrylamide concentration (Table 6.10.1) in which the multiprotein complex of interest is located on the lower third of the gel after the electrophoresis.

   The reason for this is that all "antibody shifts" will be above the protein complex.

4. Load the BN gel at 4°C as in Basic Protocol 1. Load the samples in the order of the dilution (see Fig. 6.10.2). At the end, also load the solution containing the multiprotein complex (with no antibody) as reference.

   Do not load any sample in the well next to the sample with the highest concentration of antibody. In this well, the marker mix can be loaded. If a second series of antibody-added samples is loaded, use the reverse order (see Fig. 6.10.2).

Run and analyze the Blue Native gel

5. Run NAMOS BN gels as described in the Basic Protocol 1, except reduce the voltage at the beginning of the separation; if a small gel has been prepared (e.g., using BioRad Protean II or III), run at 50 V; if a large gel has been prepared (e.g., using BioRad Protean II xi), run at 75. Continue electrophoresis at the appropriate abovementioned voltage until the sample has entered the separating gel. At that point, increase voltage to 180 V for small gel or 400 V for large gel.

6. Analyze the gels by immunoblotting (Alternate Protocol 1 or 2) using only antibodies that recognize the protein/protein complex of interest.

   The detection method should not stain the monoclonal antibodies used at steps 1 and 2. For example, for detection, use directly labeled antibodies or polyclonal rabbit antiserum in combination with labeled anti–rabbit IgG antibodies that do not cross-react with the antibodies that were used for the shift. In order to control for the cross-reactivity, also load the highest concentration of the monoclonal antibody alone, i.e., without adding the protein/protein mixture of interest.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Acrylamide/bisacrylamide mix

50 ml of 19:1 acrylamide/bisacrylamide solution
239 ml of 37.5:1 acrylamide/bisacrylamide solution
Store at room temperature (stable at least 1 year)

This will result in a ratio of 32:1 with 40% acrylamide.

BN anode buffer

Dilute 50 ml of 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl; store up to 1 year at room temperature) to 50 mM final by adding water to 1 liter.

BN cathode buffer

15 ml 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl) (15 mM final)
50 ml 1 M tricine (50 mM final)
0.2 g Coomassie blue G250 (not other Coomassie blues) (0.02% w/v final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

BN cathode buffer (low Coomassie)

15 ml 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl) (15 mM final)
50 ml 1 M tricine (50 mM final)
0.02 g Coomassie blue G250 (not other Coomassie blues) (0.002% w/v final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

BN dialysis buffer

Mix 2× BN lysis buffer stock solution (see recipe) and the detergent stock solution (see recipe) of choice so that the BN lysis buffer has a final concentration of 1× and the detergent solution has a final concentration of 0.1× (but for digitonin, use 0.3×, due to its low critical micelle concentration). Add PMSF and sodium orthovanadate (see recipe for protease and phosphatase inhibitor stock solutions) to a final concentration of 1×. Prepare fresh just before use and cool to 4°C.

Upon addition of orthovanadate, the buffer will assume a yellow color.

BN gel buffer, 3×

150 ml 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl) (150 mM final)
200 ml 1 M 6-aminohexanoic acid (Sigma-Aldrich) stock solution (200 mM final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

BN lysis buffer

Mix 2× BN lysis buffer stock solution (see recipe) and the detergent stock solution (see recipe) of choice so that both have a final concentration of 1×. Add protease and phosphatase inhibitors (see recipe) to a final concentration of 1×. Prepare fresh just before use and cool to 4°C.

Upon addition of orthovanadate, the buffer will assume a yellow color.

BN lysis buffer stock solution (without detergent and inhibitors), 2×

40 ml 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl) (20 mM final)
500 ml 1 M 6-aminohexanoic acid (Sigma-Aldrich) stock solution (500 mM final)
40 ml 1 M NaCl (20 mM final)
4 ml 0.5 M EDTA, pH 8.0 (*APPENDIX 2A*; 2 mM final)
200 ml glycerol (10% v/v final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

Preparation of an EDTA stock solution is described in UNIT 6.4.

BN stacking gel solution

3 ml 3× BN gel buffer (see recipe)
0.72 ml acrylamide/bisacrylamide mix (see recipe)
5.28 ml H_2O
120 µl 10% (w/v aqueous) ammonium persulfate (add immediately before pouring gel)
12 µl TEMED (add immediately before pouring gel)

This will result in a 3.2% solution. Adjust volumes as necessary.

Denaturing BN transfer buffer

5.81 g Tris base (48 mM final)
2.93 g glycine (39 mM final)
200 ml methanol (20% final)
1 g SDS (0.1% final)
H_2O to 1 liter
Store at 4°C (stable at least 1 year)

Destaining solution

450 ml methanol (45% v/v final)
100 ml acetic acid (10% v/v final)
H_2O to 1 liter
Store at room temperature (stable at least 1 year)

Detergent stock solutions

2× digitonin:

Prepare a 2% (w/v) solution of digitonin (Sigma-Aldrich) in water by heating to 95°C. Store in 1- to 10-ml aliquots up to 5 years (possibly longer) at −20°C.

Thawed solutions are stable at room temperature for up to 1 week. If a precipitate forms, reheat the solution to 95°C until the digitonin redissolves.

10× Brij 96:

Prepare a 10% (w/v) solution of Brij 96 (e.g., Brij 96V) in water. Store up to 1 year (possibly longer) at room temperature.

10× Triton X-100:

Prepare a 10% (w/v) solution of Triton X-100 in water. Store up to 1 year (possibly longer) at room temperature.

10× NP-40:

Prepare a 10% (w/v) solution of Nonidet P-40 (NP-40) in water. Store up to 1 year (possibly longer) at room temperature.

10× dodecylmaltoside:

Prepare a 10% (w/v) solution of dodecylmaltoside (Sigma-Aldrich) in water. Store up to 1 year (possibly longer) at 4°C.

Any nonionic detergent that has been proven to be useful for the cells and proteins of interest can be used as well.

High-percentage BN separating gel solution

5 ml 3× BN gel buffer (see recipe)
5.63 ml acrylamide/bisacrylamide mix (see recipe)
4.38 ml 70% (v/v) glycerol
42 µl 10% (w/v aqueous) ammonium persulfate (add immediately before pouring gel)
4.2 µl TEMED (add immediately before pouring gel)

This will result in a 15% solution. Adjust volumes and acrylamide/bisacrylamide concentration as necessary. Useful concentrations range from 6% to 18%.

Low-percentage BN separating gel solution

5 ml 3× BN gel buffer (see recipe)
1.5 ml acrylamide/bisacrylamide mix (see recipe)
8.5 ml H_2O
54 µl 10% (w/v aqueous) ammonium persulfate (add immediately before pouring gel)
5.4 µl TEMED (add immediately before pouring gel)

This will result in a 4% solution. Adjust volumes as necessary.

Marker mix 1

20 µl 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl)
20 µl 1 M NaCl
143 µl 70% (v/v) glycerol
5 mg ferritin (440 kDa and 880 kDa)
5 mg catalase (232 kDa)
5 mg bovine serum albumin (66 kDa and 132 kDa)
H_2O to 1 ml
Store at 4°C (stable at least 1 year)

Marker mix 2

20 µl 1 M bis-Tris stock solution (pH adjusted to 7.0 with HCl)
20 µl 1 M NaCl
143 µl 70% (v/v) glycerol
5 mg thyroglobulin (670 kDa)
5 mg aldolase (158 kDa)
H_2O to 1 ml
Store at 4°C (stable at least 1 year)

Native BN transfer buffer

5.81 g Tris base (48 mM final)
2.93 g glycine (39 mM final)
200 ml methanol (20% final)
H_2O to 1 liter
Store at 4°C (stable at least 1 year)

Pervanadate, 100×

Mix, in the following order:
50 µl 50 mM sodium orthovanadate
57 µl H_2O
15 µl 30% H_2O_2
Incubate 5 to 30 min at room temperature (becomes brownish)
Prepare fresh

Phosphate-buffered saline (PBS), 10×

152 g NaCl (130 mM final)
24 g monobasic sodium phosphate, anhydrous (10 mM final)
1600 ml H_2O
Adjust pH to 7.4 with NaOH.
Add H_2O to 2 liters
Store at room temperature (stable at least 1 year)

PBS with 1% (w/v) SDS

100 ml of 10× PBS (see recipe; 1× final)
100 ml 10% (w/v) SDS stock solution (*APPENDIX 2A*; 1% final)
Add H_2O to 1 liter
Store at room temperature (stable at least 1 year)

Protease and phosphatase inhibitor stock solutions

1000× leupeptin:
Prepare a 10 mg/ml solution of leupeptin in water. Store in 1-ml aliquots up to 5 years (possibly longer) at −20°C.
1000× aprotinin:
Prepare a 10 mg/ml solution of aprotinin in water. Store in 1-ml aliquots up to 5 years (possibly longer) at −20°C.
100× PMSF:
Prepare a 100 mM solution of phenylmethylsulfonyl fluoride (PMSF) in ethanol. Store in 1-ml aliquots up to 5 years (possibly longer) at −20°C.
100× sodium orthovanadate:
Prepare a 50 mM solution of sodium orthovanadate in water. Store up to 1 year (possibly longer) at room temperature.
100× sodium fluoride:
Prepare 1 M solution of sodium fluoride in water. Store up to 5 years (possibly longer) at room temperature.

COMMENTARY

Background Information

Most if not all proteins require binding to other proteins in order to fulfill their function. Thus, they form multiprotein complexes (MPCs). Most proteins are part of several distinct complexes, as well as being present as monomers. The abundance of distinct complexes of which a certain protein is a subunit can vary enormously. Furthermore, complexes might have different stabilities and these can change over time and space. Therefore, identifying and analyzing complexes is a difficult task.

Common techniques to study complexes such as immuno-precipitation (*UNIT 7.2*) or two-hybrid (*UNIT 17.3*) methods allow the identification of binding partners of the protein of interest; they, however, do not yield any information about the size, number, composition, and relative abundance of MPCs. A high-resolution method that resolves these problems is Blue Native polyacrylamide gel electrophoresis (BN-PAGE; Schägger and von Jagow, 1991; Schägger et al., 1994). Originally it was developed by Hermann Schägger to separate mitochondrial membrane complexes in the mass range from 10 to 10,000 kDa (Schägger and von Jagow, 1991; Schägger et al., 1994). Later, the protocol was modified for general applicability (Camacho-Carvajal et al., 2004). Since the first description of BN-PAGE, descriptions of its use in publications has increased exponentially. It has been applied successfully in nearly every area of multiprotein research—e.g., purification of complexes, determination of their size (Schägger et al., 1994; Schägger, 1995; Model et al., 2001; Dudkina et al., 2005) and stoichiometry (Schamel et al., 2005; Swamy et al., 2007), protein complex assembly (Model et al., 2001), structure determination by two-dimensional crystallization and electron microscopy (Poetsch et al., 2000), identification of multiprotein complexes with mass spectroscopy (Rexroth et al., 2003; Camacho-Carvajal et al., 2004; Millar et al.,

2005), or clinical diagnostics of human disorders (Schägger, 1995; Schägger et al., 1996).

In principle, BN-PAGE can be used to identify protein complexes in a given biological sample or to further characterize known protein complexes (or protein-protein interactions). For the second application, it is recommended to first test a series of different nonionic detergents for their effects on the extractability and stability of the protein-protein interaction of interest by co-immunoprecipitation (*UNIT 7.2*) followed by SDS-PAGE (*UNIT 6.1*). Only a successful co-purification indicates that the complex of interest is stable and abundant enough to be detected by BN-PAGE.

In BN-PAGE, the dye Coomassie blue, which binds nonspecifically to proteins and is itself negatively charged, is used. Therefore, the electrophoretic mobility of a multiprotein complex is determined by the negative charge of the bound Coomassie blue dye and the size and shape of the complex. Coomassie blue does not act as a detergent and preserves the structure of protein complexes. In contrast to other native gel electrophoresis systems, protein complexes are separated independently of isoelectric point and, therefore, the size of a complex can be estimated. In addition, the binding of Coomassie blue to proteins reduces their tendency to aggregate during the stacking step of the electrophoresis.

Following the first-dimension BN-PAGE, a number of subsequent biochemical techniques can be applied. Although proteins are already visible as blue bands after BN-PAGE, it can be useful to stain again with Coomassie blue to reach a higher detection sensitivity. Alternatively, silver staining (*UNIT 6.6*) or immunoblotting (*UNIT 6.2*) are commonly used. The individual subunits of a complex can be identified by SDS-PAGE or the Native Antibody-Based Mobility Shift (NAMOS) assay (see Basic Protocol 3). The NAMOS assay is a variant of BN-PAGE in which the stoichiometry of multiprotein complexes can be determined without purification of the complex of interest (Swamy et al., 2007).

Critical Parameters and Troubleshooting

Potential problems in pouring and running acrylamide gels in general are described in *UNIT 6.1*. These include problems in the polymerization of the gel and critical factors when using multicasting equipment.

Removing the comb of the BN stacking gel might destroy the wells of the stacking gel, due to the low percentage of acrylamide. Try to remove the comb slowly, pulling it out slightly perpendicular to the plane of the gel. This allows air to enter the wells. If this does not help, increase the acrylamide/bisacrylamide concentration of the BN stacking gel by 0.3%. If the gel pieces that form the wells are not broken, but just displaced, try to fix them with a syringe needle. Unwanted air bubbles in the stacking gel can be aspirated using a syringe. Test for leakiness with the BN cathode buffer before loading your sample.

A proper acrylamide concentration for the BN gel and the subsequent second-dimension SDS gel should be selected to optimize resolution within the desired molecular weight range. Use 4% to 7% BN gels for protein complexes of more than 1000 kDa, 4% to 10% for 500 kDa, 4% to 12% for 250 kDa, and 4% to 18% for proteins smaller than 100 kDa.

The first critical step in BN-PAGE is the preparation of the sample, since potassium and divalent cations that interact with Coomassie blue have to be removed. They are substituted by 6-aminohexanoic acid in order to maintain a certain ionic strength of the solution, which increases the solubility of many proteins. Therefore, the lysate has to be dialysed against BN dialysis buffer.

One frequent problem is that Coomassie blue and Coomassie blue–bound proteins precipitate in the gel wells, and subsequently the sample does not enter the gel. In this case, either reduce the amount of sample or improve dialysis of the sample. Use a larger dialysis reservoir volume, remove all air bubbles at the dialysis membrane, or prolong dialysis time.

Depending on the detergent, the dye front might contain peaks of detergent that prevent proteins from entering below these areas. This is pronounced when using polyoxyethylene detergents such as Brij 96 and Triton X-100. If your multiprotein complex of interest runs at a higher position than these precipitates, then this might not be a problem. Otherwise reduce the amount of detergent in the BN dialysis buffer.

To detect the protein of interest by Coomassie brilliant blue staining, it should be present in 0.5 to 2 μg amounts. If silver staining is used, this amount can be reduced to 0.1 μg. 20 to 40 μg of a protein mixture is typically loaded into a well of 50-μl volume on a 1-mm slab BN gel (16 cm, 15 wells). For immunoblotting, much less protein is required, depending on the quality of the antibody used for detection.

Instead of giving a defined band in the first dimension or a circular spot in two-dimensional BN/SDS-PAGE, a smear that was generated in the first dimension may be seen. This could have several reasons. First, the electrophoresis might not have been optimal. Try to improve dialysis of the sample (see above). If the protein migrates much higher than the dye front, a shorter electrophoresis might help. Second, the protein might have aggregated. Decrease the amount of sample loaded. Do not freeze and thaw the sample. Try to further purify the protein of interest under native conditions. Third, the protein might be present in several overlapping complexes. A two-dimensional BN/BN-PAGE could prove whether several overlapping complexes coexist that contain the protein of interest. For details and other techniques to explore this possibility, see Schamel et al. (2005).

Sometimes the protein is detected in a defined complex, but not in a reproducible manner. In this case the complex might have been an artifact generated during the electrophoresis step. This could be due to a step in the gel gradient—i.e., the gradient was not continuous. When pouring the gradient, make sure that there is a continuous flow of liquid and also that the flow is continuous between the two cylinders of the gradient maker. While loading the first-dimension gel slice onto the second-dimension SDS-PAGE, there could have been a small air bubble under the slice. This prevents entry of protein along that vertical line, giving the impression that two MPCs exist, rather than one.

If a certain interaction between two proteins is not seen by co-immunopurifications, do not expect to detect it by BN-PAGE. If the protein of interest is only found as a monomer, although it should be present in a complex, the complex could have been disrupted by detergent or be of low abundance. Thus, the choice of detergent is important in extracting but not disrupting multiprotein complexes. Without detergent, proteins tend to aggregate during the stacking step of the electrophoresis and do not enter the separating gel properly. Thus, a certain concentration of detergent has to be present in the sample. Unfortunately, general rules for the choice of detergent do not exist. Compare several detergents of different classes and three different concentrations of each in preliminary experiments, including detergents that allow successful co-immunopurifications of the proteins expected to be present in a common complex.

In the BN lysis buffer, orthovanadate is included to inhibit phosphatases. Since orthovanadate is a small molecule, it is rapidly separated from the sample during the BN-PAGE run. Since it is a reversible inhibitor, phosphatases become active once orthovanadate is removed. Thus, phosphorylation of the proteins might be lost during the native electrophoresis, where active phosphatases might be present. Since pervanadate irreversibly inhibits phosphatases, it should not be omitted from the BN dialysis buffer when phosphorylation of proteins needs to be preserved.

One common surprise that is encountered in performing BN-PAGE is that the size of the protein complex of interest does not match the expected value. First, the detergent micelle around transmembrane regions adds to the size of the corresponding protein. Hence, the size of the protein or protein complex might be larger than that obtained by simply adding the molecular weights of the individual subunits. Thus, to estimate the size of transmembrane proteins (complexes), the mass calibration markers should also be transmembrane proteins solubilized with the same detergent. Some water-soluble proteins match the calibration curve of dodecylmaltoside-solubilized transmembrane proteins, and therefore might be used as standards (Schägger et al., 1994). Second, protein glycosylation and phosphorylation might alter the running behavior of proteins in BN-PAGE. Note that the marker proteins are usually non-transmembrane, non-glycosylated, and non-phosphorylated proteins. Especially if you work with transmembrane proteins, you cannot deduce the molecular weight of the proteins from BN-PAGE. Third, in addition to their molecular weight, proteins are also separated according to their shape and number of bound Coomassie blue molecules. Thus, proteins that deviate significantly from a ball-like shape and very basic proteins show reduced mobility. Lastly, it might be that the expected molecular weight value of the protein complex is wrong.

Many antibodies that work well for the immunodetection (western blotting) of your protein of interest after SDS-PAGE do not recognize the protein after the first-dimension BN-PAGE. In the author's experience, the removal of Coomassie blue from the blotting membrane does not help. Try both conditions for the transfer to the blotting membrane (Alternate Protocols 1 and 2). If this does

not help, then a two-dimensional BN/SDS-PAGE must be performed. In any case, one should verify immunoblotting results of a first-dimension BN-PAGE by two-dimensional BN/SDS-PAGE, in order to prove that the detected protein indeed is the protein of interest and not a cross-reactivity of the antibody.

Performing a successful NAMOS assay critically depends on the quality of the antibodies used to shift the protein complex of interest (Swamy et al., 2007). Several problems could arise. First, protein complexes clustered by antibodies can be very large; thus, use Alternate Protocol 1 to disassemble these aggregates to efficiently transfer them to the blotting membrane. Second, some antibodies aggregate with themselves, thus producing a ladder-like shift pattern (Swamy et al., 2007). With these antibodies, a continuous increase in antibody concentration leads to a constant generation of new larger bands while smaller ones disappear. Thus, the stoichiometry of a multiprotein complex cannot be determined with this type of antibody; however, it is possible to determine whether all complexes contain the subunit in question. Use a fresh preparation of antibody, prepare Fab fragments, or remove antibody aggregates by ultracentrifugation. Third, it is possible that a given antibody cannot bind to all identical subunits of a multiprotein complex, probably because the complex is not symmetric, rendering the epitope accessible in one copy of the subunit but not in its neighboring copy. Likewise, if the two epitopes are very close within the complex, two antibody molecules might not be able to bind simultaneously due to steric hindrance. This type of antibody gives an underestimate of the actual stoichiometry (Swamy et al., 2007). In conclusion, not all antibodies result in the number of shifts that correspond to the number of subunit copies present in the protein complex. Thus, it is recommended to use several independent antibodies per subunit, and the Fab fragments of those antibodies.

Anticipated Results

In BN-PAGE, native protein and multiprotein complexes are separated according to their size. Thus, using two-dimensional BN/SDS-PAGE, protein complexes can be identified and characterized in terms of their relative abundance, subunit composition, and size. Since BN-PAGE is only the separation technique, the sensitivity of detection depends on the detection method used. Furthermore, the NAMOS assay allows the determination of the stoichiometries even from nonpurified complexes. Typical results are illustrated in Figures 6.10.1 and 6.10.2. Comparison of two or more samples is possible if the pouring and running of the gels was done in similar way. In combination with modern mass spectroscopy, BN-PAGE, with its high-resolution properties, is an excellent choice to identify novel protein complexes from any biological source.

Time Considerations

Preparation of the sample as described in the Support Protocol takes 6 hr or overnight. If purified proteins or organelle fractions are used, the required time can be substantially longer. However, if utilizing desalting columns to obtain samples with low cation concentrations, the time can be shorter. Since the sample should be separated immediately by BN-PAGE (and cannot be frozen and stored), one should reserve some time for gel loading, which usually takes another 0.5 to 1 hr. Depending on the gel size, running of the BN-PAGE takes from 6 hr to overnight. Loading of two second-dimension SDS-PAGE gels takes ~1 to 1.5 hr. Running the SDS-PAGE takes between 1.5 hr and overnight, again depending on the size of the gels. Thus, two-dimensional BN/SDS-PAGE takes at least 1.5 days—not counting the visualization process of the proteins of interest.

One rate-limiting step is the running of the second-dimension gels. 30 to 40 lanes can easily be separated in parallel on two BN gels, but loading, running, and detection from 30 to 40 second-dimension gels requires substantial operator time and electrophoresis equipment. Therefore, whenever possible, one should try to use only first-dimension BN gels without the need for the second dimension.

Pouring gradient gels is another time-consuming process. To minimize the time requirement, it is strongly recommended to use multicasting equipment to pour several gels at once. This also ensures best reproducibility for critical comparisons of multiple samples.

Acknowledgements

I thank Michael Reth and Klaus Pfanner for initially introducing me to BN-PAGE and Hermann Schägger for his invaluable help in setting up the technique. I further thank Balbino Alarcón for his advice on establishing the NAMOS assay and members of my laboratory for constantly improving the various BN techniques: Margarita Camacho-Carvajal, Mahima Swamy, Thomas Bock, Eszter Molnar, Susana Minguet, Elaine Pashupati Dopfer, and Gabrielle Siegers.

I also acknowledge financial support by the European Union through an individual Marie Curie fellowship and the Deutsche Forschungsgemeinschaft through the Emmy Noether program (SCHA 976/1) and the SFB620.

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Key References

Camacho-Carvajal et al., 2004. See above.

Describes the separation of cellular lysates by BN-PAGE.

Schägger and von Jagow, 1991. See above.

Describes, for first time, BN-PAGE and second-dimension BN/SDS-PAGE using solubilized mitochondria.

Swamy et al., 2007. See above.

Details the NAMOS assay with an extensive discussion of anticipated results.